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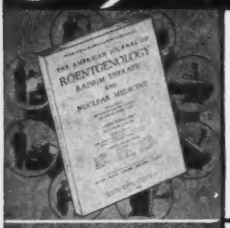

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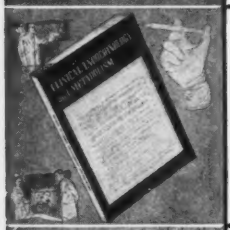

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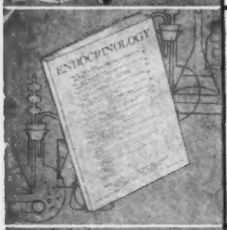

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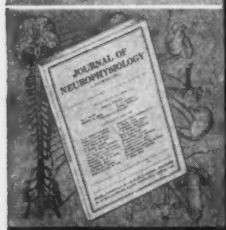

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ENDOCRINOLOGY

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ANALYSIS OF THE BLOOD SUGAR RESPONSE OF OBESE-HYPERGLYCEMIC MICE AND NORMAL MICE TO HORMONES: GROWTH HORMONE, CORTISONE, AND CORTICOTROPIN[1]

KENNETH H. SHULL[2,3] AND JEAN MAYER
with the technical assistance of DEMETRA J. SILIDES
Department of Biological Chemistry, Harvard Medical School, and Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts

INFORMATION on the effects of growth hormone, corticotropin (ACTH), or cortisone on the blood sugar of mice is scanty. Young (1938) found that an anterior pituitary extract which was diabetogenic in cats and dogs was without effect on the blood sugar when injected into mice. Westermeyer and Raben (1954) found that their oxycel preparation of corticotropin (Rabin *et al.*, 1952) produced a fall in the blood sugar of Swiss mice after administration of doses of one to 300 μg. The decrease was significant after one-half hour and maximal after 3 hours. These investigators also found that larger doses of Armour growth hormone (0.1 mg. to 5.0 mg.) produced decreases in blood sugar of similar magnitude to those produced by corticotropin.

In a systematic investigation of the biochemical and endocrine factors involved in the maintenance of hyperglycemic blood sugar levels in the obese-hyperglycemic mice, it was of importance to determine the effects of the diabetogenic hormones on the blood sugar of these animals. Previous

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[2] Part of this work performed during term of a predoctoral Public Health Service Research Fellowship, National Heart Institute, National Institutes of Health, Bethesda, Maryland.

[3] The data in this paper are taken from a thesis presented to the Division of Medical Sciences, The Graduate School of Arts and Sciences, Harvard University, in partial fulfillment of the requirements for the degree of doctor of philosophy in the subject of biochemistry.

communications dealt with the hepatic glycogen turnover (Shull and Mayer, 1955) and the levels of hexokinase, glucose-6-phosphatase, and phosphorylase (Shull *et al.* 1955) in these animals. Studies of the thryoid gland of these animals have revealed no abnormal histology or function (Goldberg and Mayer, 1952). Daily thyroxine administration over a period of a month to the obese animals produced a decrease in blood sugar, food intake, and weight (Mayer, Russell, Bates, and Dickie, 1953). Although examinations of the adrenals (Mayer and Barrnett, 1953) and the pituitaries (Andrus and Mayer, unpublished observations) of these obese mice have revealed near normal histologies, it was reasoned that the disturbances in blood sugar homeostasis produced by the diabetogenic principles from these glands might shed some light upon the mechanisms causing the hyperglycemia in the obese mice. In addition, hypo- or hypersecretion of an endocrine gland is not always accompanied by demonstrable pathology. This is sometimes the case, for example, with the islets of Langerhans in humans suffering from diabetes mellitus (Warren and LeCompte, 1952). A study of the effects of growth hormone, corticotropin and cortisone on the blood sugar of obese and non-obese mice was made. Previous reports from this laboratory (Mayer and Silides, 1953; Mayer, Andrus, and Silides, 1953) described the effects of multiple doses of growth hormone on the blood sugar of the obese-hyperglycemic mice. The present study is an investigation of the blood sugar changes produced by single doses of growth hormone, ACTH, and cortisone after various time intervals. A possible mechanism for these blood sugar changes is also presented.

METHODS

The mice used in these studies varied in age from 3 to 6 months. Equal numbers of male and female obese and their non-obese littermate controls were selected from stock cages. The animals were housed in pairs in small screen-bottomed cages during treatment and assay periods. Unless otherwise stated, all animals were fed *ad libitum* a commercial chow diet prior to and during assay periods. Blood samples of 0.1 cc. were obtained from a tail vein prior to injection of the test substance and again after the stated time interval. Blood sugar determinations were carried out in duplicate by the method of Somogyi (1945) using the colorimetric procedure of Nelson (1944). The following special procedures and methods were applied to the individual hormonal assays.

Growth Hormone:[4] Growth hormone was dissolved in physiological saline by triturating with 0.01 N NaOH until all the hormone was in solution. Two mg. contained in 0.25 cc. of solution were injected subcutaneously in all assays. One experimental group of obese mice was maintained for 2 weeks on a high fat carbohydrate-free diet (Mayer and Jones, 1953). Twenty animals which had maintained or increased their weight were selected for the growth hormone study. The obese mice used in the fasting experiment were injected with growth hormone and then started on the fast. In this way the hormone exerted its effect over the entire period of the fast. The glucose tolerance studies were performed 12 hours after the administration of growth hormone or saline. One hundred mg. of glucose were administered intraperitoneally and blood samples were ob-

[4] Lot #285-183 kindly furnished by the Armour Co., Chicago, Illinois.

tained at 0, 1, and 2-hour intervals. All control animals were injected with the alkaline saline solution used as solvent for growth hormone.

ACTH[5] *and Cortisone:* ACTH was dissolved in physiological saline by triturating with 0.01 N HCl until all of the hormone was in solution. Two and one-half mg. (2.6 units) contained in 0.25 cc. of solution were injected intraperitoneally. Cortisone in the form of a saline suspension of cortisone acetate was used for all studies. Ten mg. contained in 0.4 cc. of solution were injected intraperitoneally. In general, ACTH was not tolerated well by the obese mice. Larger doses than 2.6 units proved to be too toxic to permit assays to be carried out. Since assay periods corresponding to those used in the growth hormone study were used when assaying ACTH and cortisone, separate controls were usually not employed.

RESULTS

Table 1 lists the effect of growth hormone as well as cortisone and ACTH on the blood sugar of non-obese mice. Growth hormone did not appear to

TABLE 1. BLOOD SUGAR RESPONSE OF FED NON-OBESE MICE TO GROWTH HORMONE, CORTISONE AND ACTH AFTER VARIOUS TIME INTERVALS

	Treatment	Number of animals	Assay period (hr.)	Pretreatment blood sugar* (mg.%)	Change in blood sugar* (mg.%)
Treated	Growth Hormone	3	0.5	110 ± 15	27 ± 13
Control	Saline	4	0.5	108 ± 19	27 ± 19
Treated	Growth Hormone	6	1.0	107 ± 8	58 ± 15
Control	Saline	4	1.0	115 ± 5	32 ± 11
Treated	Growth Hormone	3	3.0	100 ± 6	61 ± 27
Treated	Cortisone	4	3.0	120 ± 13	19 ± 50
Treated	Cortisone	3	4.0	137 ± 16	11 ± 9
Treated	ACTH	3	4.0	119 ± 21	− 6 ± 18
Control	Saline	3	3.0	119 ± 5	40 ± 16
Treated	Growth Hormone	6	12.0	159 ± 14	−20 ± 18
Control	Saline	5	12.0	155 ± 14	1 ± 22

* Mean ± standard deviation.

affect significantly the blood sugar of these mice at the half-hour test period. After one hour growth hormone produced a small but significant increase in blood sugar which persisted through the three-hour test period although with decreased statistical significance. Twelve hours after receiving growth hormone the blood sugar levels of the non-obese mice appeared to be somewhat lower than the pretreatment levels, although this decrease was not of a high order of statistical significance. Neither cortisone nor ACTH appeared to produce either a significant increase or decrease in the blood sugar of the non-obese animals after test periods of 3 or 4 hours. The increase in blood sugar observed in some groups of control animals was probably due to epinephrine released during the handling and bleeding of the animals.

The effects of growth hormone, ACTH, and cortisone, on the blood sugar of the obese animals are shown in Table 2. Inspection of these data

[5] Lot #84-5-HS1 kindly furnished by Armour Co., Chicago, Illinois.

revealed that in contrast to the non-obese animals growth hormone produced a hypoglycemic response in the obese animals after one hour. This hypoglycemia became significantly greater after 3 hours but had completely disappeared after 6 hours. At the latter assay period growth hormone did not produce an over-all significant increase in blood sugar, but 2 of the 8 animals assayed did show significant increases in blood sugar, viz. increases of 100 mg.% and 183 mg.%. These increases became obscured in the mean value by the lack of response of the other animals. Twelve hours after growth hormone treatment all obese animals exhibited marked hyperglycemic responses. It is worthy of emphasis that the magnitude of this increase in blood sugar, 204 ± 72 mg.%, was considerably greater than

TABLE 2. BLOOD SUGAR RESPONSE OF FED AND FASTED OBESE MICE TO GROWTH HORMONE, ACTH, AND CORTISONE AFTER VARIOUS TIME INTERVALS

	Diet	Treatment	Number of animals	Assay period (hr.)	Pretreatment blood sugar* (mg.%)	Change in blood sugar* (mg.%)
Treated	Chow	Growth Hormone	3	1.0	297 ± 79	−32 ± 48
Control	Chow	Saline	3	1.0	320 ± 173	21 ± 39
Treated	Chow	Growth Hormone	6	3.0	287 ± 136	−43 ± 40
Control	Chow	Saline	3	3.0	166 ± 42	35 ± 45
Treated	Chow	Cortisone	8	3.0	226 ± 116	−31 ± 52
Treated	Chow	ACTH	5	4.0	128 ± 23	−26 ± 23
Treated	Chow	Growth Hormone	8	6.0	228 ± 90	29 ± 75
Treated	Chow	Growth Hormone	17	12.0	202 ± 47	204 ± 72
Treated	Chow	Cortisone	6	12.0	332 ± 114	135 ± 55
Treated	Chow	ACTH	6	12.0	342 ± 153	− 9 ± 50
Control	Chow	Saline	8	12.0	267 ± 92	25 ± 85
Treated	Fasted	Growth Hormone	13	12.0	244 ± 127	−65 ± 55
Control	Fasted	Saline	13	12.0	192 ± 55	−37 ± 67
Treated	CHO-Free†	Growth Hormone	13	12.0	145 ± 85	76 ± 81
Control	CHO-Free†	Saline	7	12.0	248 ± 123	− 5 ± 32

* Mean ± standard deviation.
† High fat carbohydrate-free diet.

the significant blood sugar increase, 58 ± 15 mg.% exhibited by the non-obese animals. The statistical significance of this increase in blood sugar shown by the obese animals 12 hours after the administration of growth hormone was extremely high ($p < 0.001$) when compared either with the obese saline controls or with the growth hormone treated non-obese animals assayed at one hour. Percentage-wise, the increase in blood sugar of the obese animals represented a $105\% \pm 43\%$ increase over the already hyperglycemic pre-treatment level of 202 ± 47 mg.%. The effects of cortisone and ACTH on the blood sugar of obese animals after periods of 3, 4, and 12 hours are also shown in Table 2. These results indicated that ACTH did not significantly change the blood sugar of the obese mice at the test periods studied. However, cortisone acted similarly to growth hormone; it caused a decrease in the blood sugar after 3 hours and produced a marked hyperglycemic response in 12 hours. These responses were not as great as those observed with growth hormone treatment nor of as high a statistical

significance. Nevertheless, taken together with the growth hormone responses, they indicated a marked impairment in the obese mice in their adaptation to these diabetogenic agents.

In order to determine the influence of food intake and carbohydrate alimentation upon the growth hormone-induced hyperglycemic responses in the obese mice, a group of 26 obese mice were injected with growth hormone and fasted; 12 hours later they were assayed. Another group of 20 obese mice subsisting on a carbohydrate-free high fat diet were injected with growth hormone and also assayed 12 hours later. The results of these experiments are presented in Table 2. Inspection of the data obtained from the growth hormone-treated fasted animals indicated that lack of food for a 12 hour period had completely abolished the hyperglycemic response. Further comparisons of the saline treated control value with that of the growth hormone treated value suggested that growth hormone may possibly have caused a decrease in blood sugar over and above that caused by fasting. Examination of the mean blood sugar increase produced by growth hormone when administered to obese mice on a carbohydrate-free regimen indicated that carbohydrate intake was important in the blood sugar response. While these animals showed a significant increase in blood sugar when compared to their controls, this increase was very significantly less ($p < 0.001$) than that exhibited by growth hormone treated obese animals maintained on the carbohydrate-containing chow diet.

Since the hyperglycemic effect of growth hormone in the obese mice appeared to depend upon carbohydrate alimentation for its maximal effect, glucose tolerance tests were conducted on a series of animals 12 hours after treatment with growth hormone or saline. The results of these tolerance tests are shown in Table 3. These data indicated that the blood sugar of the growth hormone-treated animals was elevated to a significantly higher level than control animals one hr. after the test dose of glucose, and that the blood sugar of the former group had remained at a level very significantly higher than the latter group 2 hours after the administration of glucose. The blood sugar of the control group approached pretreatment levels in 2 hours while the growth hormone-treated group remained approximately 100 mg.% above pretreatment level. These results together with those obtained on fasted animals and animals on carbohydrate-free diets

TABLE 3. THE INFLUENCE OF GROWTH HORMONE ON THE GLUCOSE TOLERANCE OF OBESE MICE

	Assay period (hr.)	Number of animals	Change in blood sugar after 1 hr.* (mg.%)	Change in blood sugar after 2 hr.* (mg.%)
Treated	12	6	171 ± 65	99 ± 32
Control	12	12	101 ± 57	9 ± 57

* Mean ± standard deviation.

strongly indicated that the growth hormone-induced hyperglycemic response was due, at least in part, to a decreased peripheral uptake of glucose.

DISCUSSION

The hyperglycemic response produced by growth hormone in the obese-hyperglycemic mice differed not only in magnitude but also in time of appearance from that produced in the non-obese mice. The non-obese mice showed a small but significant increase in blood sugar one hour after growth hormone administration which persisted throughout the three-hour assay period, although with diminishing statistical significance. The blood sugar 12 hours after growth hormone treatment was not only no longer elevated but had reached hypoglycemic levels compared to pretreatment values. In contrast, the obese mice exhibited a significant blood sugar decrease in one to 3 hours and did not begin to show an increase until 6 hours after the administration of growth hormone. At the six-hour test period 2 out of 8 animals showed an increased blood sugar in response to growth hormone. The impaired glucose tolerance together with the absence of response in fasted animals and the diminished response of animals on carbohydrate-free diets was evidence that growth hormone caused a decreased peripheral uptake of glucose. These facts did not exclude the possibility that factors other than peripheral inhibition of glucose uptake contributed to the growth hormone induced hyperglycemia.

It is of importance to note that the hyperglycemic response of the obese-hyperglycemic animals to growth hormone is not seen in mice made obese by gold thioglucose (Mayer and Zighera, 1954) nor in mice made obese by hypothalamic lesions (Mayer *et al.*, 1955). In these types of obese mice, growth hormone produces no hyperglycemia and it is, therefore, inferred that the hyperglycemic response produced in the obese-hyperglycemic mice is unique and not a characteristic of obesity *per se*.

The blood sugar changes produced by cortisone in the obese mice, although smaller in magnitude, were qualitatively similar to those produced by growth hormone in these animals. It is probable that the changes produced by cortisone are amenable to the same explanation applied to those produced by growth hormone. Although part or all of the hyperglycemic response produced by cortisone after 12 hours in the obese mice may have been due to gluconeogenesis, the end result would be no different than a hyperglycemic response produced by inhibition in glucose uptake. The failure of ACTH to induce blood sugar changes in the obese mice even though it exerts its effects through the adrenal cortex may indicate that cortisone is not a normal secretory product of the adrenals of mice.

The near normal histology presented by the adrenals and the pituitaries of the obese mice together with the failure of ACTH to induce a hyperglycemic response made it improbable that either ACTH or the hormones

of the adrenal cortex were involved in the etiology of the diabetes displayed by the obese-hyperglycemic mice. Similar considerations of the pituitary together with the normal protein mass of the obese mice in the adult state indicated that growth hormone probably was not hypersecreted by these mice.[6] The possibility, however, remains that cortisone and growth hormone might affect the same mechanism(s) responsible for the spontaneous hyperglycemia observed in the untreated animals.

SUMMARY

Growth hormone administration to the obese-hyperglycemic mice produced a 204 mg.% increase in blood sugar 12 hours after treatment. Cortisone produced an increase in blood sugar of 135 mg.% at the same assay period while corticotropin did not significantly affect the blood sugar at any assay periods studied. At short test periods of one to 3 hours growth hormone and cortisone produced small hypoglycemic responses. The glucose tolerances of the obese mice after growth hormone treatment were markedly impaired. Fasting completely abolished the growth hormone response and carbohydrate-free regimens significantly lowered the response. The non-obese controls showed a small but significant increase in blood sugar one to 3 hours after receiving growth hormone which was commpletely abolished after 12 hours; cortisone and corticotropin produced no significant change in blood sugar.

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[6] Unpublished observations with L. Nay indicated that hypophysectomy causes weight loss, a low blood sugar, and appears more lethal in the obese mice than the non-obese controls.

EFFECT OF POTASSIUM THIOCYANATE ON FETAL RETENTION OF RADIOIODINE[1]

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IN A recent study by Rugh and Booth (1954) of the effect of treatment of the pregnant mouse with thiouracil, methimazole, and thyrotrophic hormone prior to the injection of radioiodine, it was demonstrated that the expected radioiodine damage to both the mother and the fetus (Rugh 1951) was modified. Thiouracil particularly was found to protect both the maternal and fetal thyroids against radiation-induced necrosis.

Potassium thiocyanate (KSCN) has been used clinically to alter radioiodine absorption and retention by the thyroid gland (Stanley and Astwood 1949; Beierwaltes 1950; Reveno and Rosenbaum 1950; Bartels and Sjogren 1951; Stone *et al.*, 1952). It markedly inhibited I^{131} uptake by rat thyroids (Williams *et al.*, 1950). The present study was directed toward the fetus. The thiocyanate was injected into the pregnant mouse before, simultaneously with, or after the injection of radioiodine (NaI^{131}) and the effect on radioiodine retention by the mother and the fetus was determined. Gestation days from 17.5 to 19.5 were studied because they included the time when the fetal thyroid normally becomes active (Gorbman and Evans 1943; Speert *et al.*, 1951; Waterman and Gorbman 1953).

MATERIALS AND METHOD

CF_1 female mice of proven fertility were mated with males of the same strain. On gestation days 17.5 through 19.5, pregnant mice were selected for treatment.

Carrier-free radioiodine (NaI^{131}) was obtained directly from Oak Ridge and was made up in a concentration of 250 μc./cc. of distilled water. A standard dose of 250 μc. was injected intraperitoneally into each experimental mouse.

Potassium thiocyanate (KSCN) was made up as a 0.1% solution in 0.9% (physiological) saline, of which 1 cc. was injected (i.p.) into each experimental mouse. Control pregnant mice were injected with the radioiodine alone, without thiocyanate. The injections of potassium thiocyanate were made either 4 hours prior to the I^{131}, simultaneously, or 4 hours after the radioiodine.

The radioactivity of each mouse (pregnant mouse or mouse with and without its litter) was determined by means of a Geiger-Müller counter. The mouse was placed in a covered plastic fly box which was 2 cm. high and had a diameter of 6.5 cm. and was kept in constant geometrical relation to the counter tube. At least two background readings were taken before and again after the readings for the experimental animals, and the

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PERCENTAGE RADIOIODINE RETAINED AT VARIOUS INTERVALS

	Controls			17.5 days				18.5 days						19.5 days								
	24 hrs.	48 hrs.	96 hrs.	24 hrs.	96 hrs.			24 hrs.			48 hrs.			24 hrs.			48 hrs.			96 hrs.		
	Total	Total	Total	Total	T	♀	L	T	♀	L	T	♀	L	T	♀	L	T	♀	L	T	♀	L
Controls	17.3	13.3		22.7	10.3	7.6	2.7	28.2	17.7	10.5				30.1	15.2	14.9	18.1	12.1	6.0	11.1	8.2	2.9
	11.0	9.0	6.4	20.6	9.1	6.6	2.5	28.2	11.9	16.3				47.8	23.9	23.9	27.4	15.0	12.4	15.7	9.0	6.7
	8.1	6.4	4.9	35.2	11.8	6.9	4.9	50.3	13.5	36.8	23.1	9.2	13.9	40.3	18.4	21.9	19.2	12.0	7.2	8.3	4.3	4.0
	19.5	13.8	5.7	25.1	5.8	2.2	8.0	48.1	16.3	31.8	22.7	8.2	14.5	50.6	11.8	38.8	45.4	12.1	33.3	17.5	6.2	11.3
Av.	14.0	10.6	5.7	25.9	9.8	6.7	3.1	38.7	14.9	23.9	22.9	8.7	14.2	42.2	17.3	24.9	27.5	12.8	14.7	13.2	6.9	6.2
Pre-KSCN	9.2	6.8		18.7	8.5	6.1	2.4	9.9	8.4	1.5				4.2	2.4	2.0	2.7	1.8	0.9	1.8	1.2	0.6
	4.1	3.3		5.8	3.2	2.0	1.2	11.3	10.0	1.3				14.3	4.4	9.9	9.0	2.9	6.1	4.2	1.9	2.3
														6.2	3.6	2.6	4.6	2.4	2.2	2.2	1.0	1.2
														7.4	3.5	3.9	4.9	2.7	2.2	4.6	3.4	1.2
Av.	6.7	5.1		12.3	5.9	4.1	1.8	10.6	9.2	1.4				8.3	3.5	4.6	5.3	2.5	2.9	3.2	1.9	1.3
Simultaneous KSCN	13.9	11.3						6.7	5.6	1.1				16.0	3.2	12.8	10.8	3.4	7.4	3.7	1.9	1.3
	7.5	5.9	5.3					16.7	12.4	4.3				6.4	5.1	1.3	4.9	3.3	1.6	2.5	2.2	0.3
	7.5	5.6	5.0											27.3	13.9	13.4	14.8	10.1	4.7	8.5	6.3	2.5
Av.	9.6	7.6	5.2					11.7	9.0	2.7				16.6	7.4	9.2	10.2	5.6	4.6	4.9	3.5	1.5
Post-KSCN	9.9	7.6	5.1	11.6	8.4	6.3	2.1	13.7	11.6	2.1				13.8	9.1	2.8	6.8	4.4	2.2	6.6	4.7	1.9
	8.0	5.4		8.0	4.9	2.9	2.0	7.1	3.0	4.1				11.2	8.4	2.8	7.8	5.4	2.4	3.8	3.4	0.4
								10.7	7.4	3.4	3.8	3.0	1.8	13.4	8.3	5.1	7.0	4.3	2.7	3.6	2.5	1.1
								14.0	10.4	3.6	9.3	5.3	4.0									
								17.5	10.8	6.7	9.6	4.5	5.1									
Av.	9.0	6.5	(5.1)	9.8	6.7	4.6	2.1	12.6	8.6	4.0	7.9	4.3	3.6	12.3	8.2	4.1	7.7	4.9	2.8	4.7	3.5	1.2

NOTE: All readings are net, taking into account the background radioactive decay is independent of location, in mother or litter.
Key: T = Total. ♀ = Mother only. L = Litter only.

values were deducted from the data recorded. There was never any contamination of the receptacles. In most cases readings were taken at 0, 24, 48, and 96 hours after the injection of radioiodine. Following delivery, readings were taken separately of the mother and of her litter. In each case two 4 minute counts were taken and averaged to determine the counts per minute. The radioactivity of the mouse immediately following injection was designated as 100% activity and subsequent counts were expressed as percentages of this original value. In each calculation the loss of radioactivity from natural decay (8 day half-life) and the background were taken into consideration, and the values recorded are net values.

EXPERIMENTAL DATA

From an examination of the percentage iodine retention, it is apparent in every case, including the male controls, and on each gestation day studied, that a higher per cent of I^{131} is retained in animals receiving no KSCN than is observed in any of the injected animals. In other words, KSCN given before, simultaneously with, or after I^{131} reduces the amount of radioiodine retained. Although retention of radioiodine in the pregnant mouse is normally considerably higher than that of control males, the KSCN-injected pregnant females show retention roughly equivalent to that of KSCN-injected males. This indicates that both females and their litters exhibit marked reduction in retention associated with KSCN administration. In uninjected pregnant and lactating females, I^{131} tends to be divided equally between mothers and litters at delivery, or else a disproportionately large amount goes to the litter. The proportion in the nursing offspring seems to fall in comparison to that in their mothers. In KSCN-treated animals, concentration in the litter was almost always smaller than that in the mothers at the time of delivery, and the proportional retention in the litters seemed to fall somewhat at later counts. Except in two cases out of 24, the treated litters exhibited smaller retention of I^{131} than their mothers at every count excluding those cases where total retention had fallen below 5% for both mother and litter. This would appear to indicate a greater protective effect of KSCN against radioiodine retention upon the litters than upon their mothers.

DISCUSSION

Potassium thiocyanate has been used clinically to hasten the excretion of radioiodine (I^{131}) from the thyroid gland. Stanley and Astwood (1947, 1949) administered the drug to human patients after inhibition of function of the thyroid gland by mercaptoimidazole and subsequent I^{131} treatment. When collection of the isotope was at a maximum, KSCN was given. They believed that the iodine thus excreted was in the form of the free iodide ion; organically bound iodine was thought not to be affected by such treatment.

Other investigators (Rawson *et al.*, 1944, 1948; Wolff *et al.*, 1946; VanderLaan *et al.*, 1946, 1947; and McGinty *et al.*, 1948) have considered

the effect of KSCN upon retention of I^{131} in laboratory animals. Although Rawson *et al.* reported that pretreatment of thyroids in rats and chicks caused greater I^{131} retention, later work (McGinty 1948, 1949; VanderLaan 1946, 1947) has raised some question with regard to their results. McGinty (1948), giving KSCN at various intervals from 1 to 24 hours before I-131, found that it blocked uptake of the isotope, with minimum retention at 1 hour before, and found that the drug was ineffectual if given 24 hours before I^{131}. VanderLaan *et al.* (1947) found the treatment effective when the KSCN was given 3 to 4 hours before I^{131} with a marked drop in efficacy at 5 hours before.

Our data, using the 4-hour interval between injections of the KSCN lowers I^{131} retention in the mouse thyroid. The control males, pre-injected with KSCN, show an average retention at 24 hours of 6.7% as compared to 13.9% for the male controls receiving I^{131} alone. Those treated either simultaneously or 4 hours after I^{131} likewise show reduction (7.5 and 9.0% respectively). The data obtained at 48 and 96 hours continue to show the same relative values as those obtained at 24 hours. The same trend is likewise demonstrated in pregnant mice, with retention of I^{131} increasing as the injection of KSCN is delayed.

Our work with pregnant mice shows a much greater influence of KSCN upon I^{131} excretion rate than does that with control males. It has previously been proved (Rugh 1951) that iodine retention by the pregnant or lactating mouse is normally greater than by a non-pregnant or non-lactating individual. The pregnant mouse without KSCN retains more I^{131} than does a non-pregnant mouse, and the radioactivity tends to be divided equally between the mother and the litter at birth (Rugh and Booth 1954). In these present data the same holds true in controls at 24 hours. In the KSCN-injected mice, the distribution of iodine in the mothers tended to exceed that in the litters at birth. Thus, while there were several exceptions, in most instances there was a differential effect of KSCN protecting the litter to a greater degree than the mother, the proportional distribution being fairly constant.

These observations may be explained in any one of several ways. *First*, the litters might be protected by KSCN acting upon the mother in such a way as to cause her to retain or excrete most of the I^{131}. *Second*, KSCN might pass through the placenta to the unborn litters more readily than it is retained by the mother, building up a resistance to I^{131} within the fetal mice. A *third* possibility is that the concentration of KSCN used has a more potent effect on the fetal than on the adult thyroid. *Fourth*, since the weight of the litter and their thyroid glands is considerably less than the weight of the mother or her thyroid, this quantitative differential may well account for the disparity in the results. In any case, a distinct lowering of the I^{131} concentration in both mother and litter is noted after KSCN administration to the pregnant female.

SUMMARY AND CONCLUSIONS

Radioiodine uptake in the mouse thyroid has been demonstrated to be inhibited by the administration of potassium thiocyanate, whether the time of injection is 4 hours before, simultaneous with, or 4 hours after I^{131} injection.

In the pregnant mouse, where retention of the isotope is normally higher than in control males, the thiocyanate injections decrease retention to a greater degree, so that resulting concentrations are approximately equal to retention in the KSCN-treated males. The thiocyanate is capable of reducing not only maternal I^{131} concentrations, but that of the unborn fetuses as well. The injected KSCN appears to have a slightly greater protective effect for the fetuses than for the pregnant female. It is postulated that the drug passes through the placenta and enters the fetal thyroid before the iodine has been bound organically and there acts in the same way as it does in adult thyroids.

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STUDIES ON THE THYROIDAL ACCUMULATION OF RHENIUM IN THE RAT[1]

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INTRODUCTION

ALTHOUGH it has been known for some years that the thyroid gland markedly concentrates iodine, it is becoming apparent, through the use of radioactive isotopes, that the thyroid gland shows a certain affinity for other members of the seventh periodic group of elements; namely, bromine, astatine, manganese, technetium and rhenium (Perlman, *et al.*, 1941, Hamilton and Soley 1940, Baumann and Metzger 1949, Baumann *et al.*, 1949a, Baumann *et al.*, 1949b, Baumann *et al.*, 1951, Bruner *et al.*, 1953, and Baumann *et al.*, 1953). The present report is concerned with the capacity of the thyroid gland to accumulate Re^{188}.

MATERIALS AND METHODS

Radioactive rhenium used in this study was produced by irradiating stable rhenium in the form of metallic powder for 24 hours in the Brookhaven reactor. The irradiated material was then dissolved in nitric acid and diluted with distilled water to give the desired specific activity. Since the material was utilized the same day it was produced, advantage was taken of the shorter half-life isotope (Table 1) and the standard volume (0.1 cc.) prepared for each intreparitoneal injection contained approximately 2 μc of radioactivity and 1 μg. of rhenium. I^{131} [2] was used as a control isotope.

All rats utilized were young Sprague-Dawley[3] males housed in a temperature-controlled room (72° ± 2° F.) and maintained on a modification[4] of the Remington low iodine diet and tap water (to which additions were sometimes made) until sacrifice. The animals were sacrificed under light ether anesthesia, approximately 2 cc. of blood were collected by a heparinized syringe from the abdominal aorta and transferred to a centrifuge tube. Approximately 0.5–1.0 cc. of plasma was prepared and saved for isotope assay. The thyroid gland was removed, weighed to the nearest 0.02 mg. on a torsion balance and placed in a vial containing 2 cc. of Bouin's fluid. The thyroid glands and plasma were counted in a well-type scintillation counter against 3 dilutions of the isotope solution as prepared for injection. The radioactivity was such that the probable error of counting did not exceed 3%.

Twenty 31 day old rats that received the low-iodine diet and 0.2 per cent thiouracil in the drinking water for eight days were used for a 1, 3, 8 and 20-hour Re^{188} uptake

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[2] I^{131} was procured from the Oak Ridge National Laboratory, Oak Ridge, Tennessee.

[3] Purchased from Sprague-Dawley, Inc., Madison, Wisconsin.

[4] Each 1000 grams of diet contained: Wheat gluten flour 180 grams; corn meal 780 grams; 20 grams Brewers' Yeast, 10 grams calcium carbonate and 10 grams sodium chloride.

TABLE 1. DECAY SCHEME FOR Re^{188} AND Re^{186}

Isotope	Half-life	Radiation		Stable daughter
		β mev	*gamma* mev	
Re^{188}	17 hours	2.2 (26%) 1.9 (62%) 1.5 (7%) 0.4 (5%)	0.155	Os^{188}
Re^{186}	91 hours	1.1 (73%) 0.9 (27%)	0.164 0.138 0.123	Os^{186}

TABLE 2. THE THYROID AND PLASMA CONTENT OF Re^{188} AND I^{131} AS A FUNCTION OF TIME BETWEEN ISOTOPE ADMINISTRATION AND SACRIFICE. THIRTY-ONE DAY OLD MALE SPRAGUE-DAWLEY RATS MAINTAINED ON LOW IODINE DIET FOR 8 DAYS WITH AND WITHOUT THIOURACIL (0.2%) ADDED TO DRINKING WATER. MEAN AND STANDARD ERROR REPORTED.

Treatment	Hrs. post-isotope	N	Body weight (gm.)	Thyroid weight (mg.)	Per cent of administered Re^{188} found in sample			Ratio	Per cent of administered I^{131} found in sample			Ratio
					Thyroid gland	Thyroid per gm.	Plasma per cc.	Thyroid (gm.) / Plasma (cc.)	Thyroid gland	Thyroid per gm.	Plasma per cc.	Thyroid (gm.) / Plasma (cc.)
Control	1	10	59±2	7.9±0.3	2.60±.23	316±21	2.20±.25	153±29	23.5 ±3.0	3,158±300	1.52±.15	2,174± 390
Thiouracil	1	10	57±1	16.1±0.9	2.13±.36	138±32	2.22±.28	63±12	3.72±0.82	229± 42	2.40±.23	95± 14
Control	3	10	58±1	8.4±0.5	0.71±.10	96±23	1.52±.32	70±17	59.1 ±5.4	5,690±470	0.50±.06	11,640±1,030
Thiouracil	3	10	58±2	18.0±0.6	1.84±.31	106±19	1.49±.07	72±14	3.43±1.16	183± 10	1.83±.18	94± 18
Control	8	10	58±1	9.8±1.1	0.32±.05	31± 2	0.43±.04	74± 8	62.9 ±4.1	6,690±240	0.25±.04	28,400±4,700
Thiouracil	8	10	59±2	20.8±1.4	0.43±.21	24± 8	0.53±.08	50±20	1.47±0.26	70± 15	0.96±.13	71± 11
Control	20	10	58±1	8.5±0.4	0.04±.02	5± 2	0.08±.02	62± 2	53.2 ±2.1	6,270±550	0.46±.10	15,100±2,110
Thiouracil	20	10	59±2	18.3±1.1	0.15±.02	8± 1	0.12±.01	71±12	0.47±0.15	24± 6	0.32±.07	74± 16

study. A like number of rats were treated as above, except that thiouracil was not added to the water.

Thirty-five 31-day-old rats that had been on the low iodine diet for 7 days were divided into 7 groups of equal numbers and given a 0.1 cc. subcutaneous injection of 1, 5 or 25 μg. of iodide (as KI) or 1, 5 or 25 μg. of rhenium (made by dissolving metallic rhenium in nitric acid+distlled water), or a control injection of distilled water. One hour later they were given Re^{188} and sacrificed one hour after isotope administration.

To glass homogenizer tubes containing 1 cc. of 1N NaOH, one of the following additions were made: Re^{188}, Re^{188} plus 5 thyroid glands from 31 day old rats maintained on the low iodine diet for 8 days, 5 thyroid glands from 31 day old rats treated as the previous group, except that a tracer dose of Re^{188} was given one hour before sacrifice or 5 thyroid glands from rats treated as the previous group, except that thiouracil (0.2%) was added to the drinking water. The contents were fractionated with zinc sulfate by a method used previously [Shellabarger 1954] and the protein and non-protein fractions assayed for radioactivity.

Twenty 32-day-old rats that had been on the low iodine diet and tap water for 7 days were given the following treatments: 5 rats received 2 mg. of KSCN in a subcutaneous injection one hour before receiving isotope administration, 5 rats received 20 mg. of sulfadiazine[5] by the same route one hour before isotope administration, 5 rats received 6 mg. of propylthiouracil by the same route one hour before isotope administration and 5 rats received no previous treatment other than the low iodine diet. Re^{188} was administered with sacrifice one hour later and radioactivity of the thyroid gland and plasma was determined.

Five 45-day-old rats that had been on the low iodine diet and tap water for 22 days received Re^{188}. They were sacrificed 20 hours later and the plasma and thyroids handled as before. Two animals were surveyed for isotope content of 15 tissues. A portion of each tissue or the whole tissue depending upon its size was weighed and assayed for radioactivity content.

RESULTS

It was apparent that a certain amount of Re^{188} was accumulated by the thyroid gland. However, when the radioactivity of the thryoid gland was corrected for the weight of the gland and compared to the radioactivity of the plasma it was apparent that the thyroid gland selectively accumulated Re^{188} as well as I^{131} (Table 2). When these data were plotted against the interval between isotope administration and sacrifice, the following points were noted: 1. Much more I^{131} than Re^{188} was accumulated by the gland. The advantage in favor of I^{131} increased from approximately 10 at one hour post-isotope administration to approximately 500 at 20 hours following isotope administration (Figs. 1 and 2). These values were in general agreement with values reported by Baumann *et al.* (1949a) for similar time intervals. 2. Chronic thiouracil treatment reduced I^{131} uptake but had no effect on or increased Re^{188} uptake. These results were similar to the effects of thiouracil on the throidal accumulation at At^{211} and will be commented on in some detail in a later section. 3. The shape and magnitude of thyroidal accumulation I^{131} curves of thiouracil-treated animals was not dissimilar to the shape and magnitude of the non-thiouracil treated Re^{188} curve.

[5] Sulfadiazine sodium, E. R. Squibb & Sons.

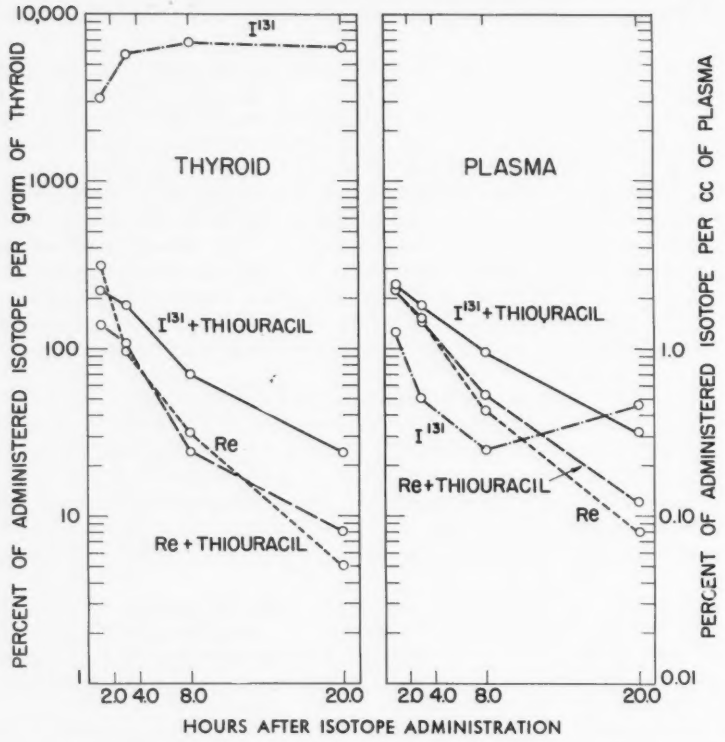

FIG. 1. Data from Table 2 plotted as per cent of administered isotope per gram of thyroid or per cc. of plasma (log basis) against hours between isotope administration and sacrifice.

Propylthiouracil, 6 mg. given one hour before a one-hour uptake study lowered the amount of I^{131} accumulated by the gland and reduced the percentage of administered isotope that was found in the protein-bound fraction. When Re^{188} was studied, propylthiouracil was found to have no effect on the accumulation of the isotope and, furthermore, no significant amount of Re^{188} was found in the protein-bound fraction of either goitrogen or non-goitrogen treated glands (Table 3).

Stable iodide, in the amount of 1, 5 or 25 μg. given one hour before a one-hour uptake study, depressed the thyroidal accumulation of I^{131} and Re^{188} while similar amounts of stable rhenium had no effect on either I^{131} or Re^{188} accumulation (Table 4).

Propylthiouracil, KSCN and sulfadiazine were effective in lowering the thyroidal accumulation of I^{131}. When the accumulation of Re^{188} was studied, only KSCN lowered accumulation and there was a suggestion of a slightly increased thyroidal accumulation of Re^{188} when the animals were pre-treated with propylthiouracil or sulfadiazine (Table 5).

When the distribution of I^{131} and Re^{188} was studied in various tissues at

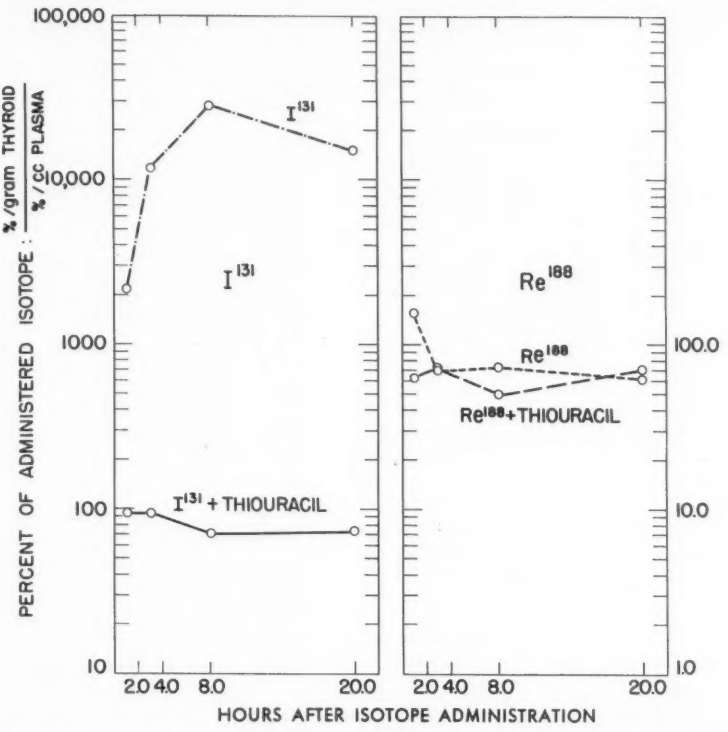

FIG. 2. Data from Table 2 plotted as the ratio of per cent of administered isotope per gram of thyroid to per cent of administered isotope per cc. of plasma (log basis) against interval between isotope administration and sacrifice.

20 hours after isotope administration, the values were in agreement for the two isotopes with two exceptions. As expected, higher thyroid I^{131} values were observed and there seemed to be more Re^{188} than I^{131} in the stomach. No significant amounts of either isotope were found in any organ or tissue other than the thyroid gland or the gastrointestinal tract (Table 6). Since

TABLE 3. THE EFFECT OF A 6 MG. DOSE OF PROPYLTHIOURACIL UPON THE THYROIDAL ACCUMULATION OF I^{131} AND Re^{188}. THIRTY-ONE-DAY-OLD MALE SPRAGUE-DAWLEY RATS MAINTAINED ON A LOW-IODINE DIET FOR 8 DATS, PROPYLTHIOURACIL GIVEN AS A SINGLE SUBCUTANEOUS INJECTION ONE HOUR BEFORE ISOTOPE ADMINISTRATION WITH SACRIFICE ONE HOUR LATER. MEAN VALUE OF PER CENT OF ADMINISTERED ISOTOPE FOUND IN SAMPLE AND THE PERCENTAGE OF THIS VALUE FOUND IN PROTEIN FRACTION OF GLAND REPORTED

Treatment	Route of isotope administration	N	Per cent of administered isotope found in sample						Per cent of radioactivity in thyroid that was found in protein fraction	
			Thyroid gland		Thyroid protein fraction		Thyroid non-protein fraction			
			Re^{188}	I^{131}	Re^{188}	I^{131}	Re^{188}	I^{131}	Re^{188}	I^{131}
Control thyroid	in vivo	10	3.48	16.88	0.53	15.66	2.95	1.22	15	93
Propylthiouracil Thyroid	in vivo	10	2.15	4.11	0.38	0.68	1.77	3.43	18	16
Control Thyroid	in vitro	1	—	—	—	—	—	—	10	13
Blank	in vitro	1	—	—	—	—	—	—	17	14

TABLE 4. THE EFFECTS OF 1, 5 OR 25 μG. OF EITHER KI (AS IODIDE) OR RHENIUM (DISSOLVED IN NITRIC ACID) UPON ONE HOUR THYROIDAL ACCUMULATION OF EITHER I^{131} OR Re^{188}. THIRTY-ONE-DAY-OLD MALE SPRAGUE-DAWLEY RATS MAINTAINED ON LOW IODINE DIET FOR 7 DAYS. STABLE ELEMENT GIVEN IN A SINGLE SUBCUTANEOUS INJECTION ONE HOUR BEFORE ISOTOPE ADMINISTRATION. MEAN AND STANDARD ERROR REPORTED

Treatment	N	Body weight (gm.)	Thyroid weight (mg.)	Per cent of administered Re^{188} found in sample			Ratio	Per cent of administered I^{131} found in sample			Ratio
				Thyroid gland	Thyroid per gm.	Plasma per cc.	Thyroid (gm.) / Plasma (cc.)	Thyroid gland	Thyroid per gm.	Plasma per cc.	Thyroid (gm.) / Plasma (cc.)
None	10	58 ± 1	7.7 ± 0.3	2.16 ± .14	279 ± 10	2.40 ± .32	125 ± 31	13.06 ± 1.24	1,725 ± 190	1.79 ± .16	982 ± 42
1 μg. rhenium	10	58 ± 1	8.8 ± 0.5	2.08 ± .34	239 ± 40	2.67 ± .51	95 ± 15	17.46 ± 1.80	1,979 ± 190	1.78 ± .05	1,129 ± 140
5 μg. rhenium	10	57 ± 1	8.2 ± 0.7	2.02 ± .36	285 ± 37	2.52 ± .16	116 ± 20	13.97 ± 2.36	1,499 ± 316	2.03 ± .17	731 ± 115
25 μg. rhenium	10	57 ± 1	8.0 ± 0.4	2.09 ± .26	287 ± 20	2.29 ± .16	126 ± 8	11.41 ± 2.44	1,295 ± 276	1.95 ± .16	711 ± 208
1 μg. KI	10	58 ± 2	9.0 ± 0.4	1.17 ± .08	137 ± 7	2.91 ± .40	50 ± 9	6.10 ± 0.85	640 ± 88	2.35 ± .28	271 ± 34
5 μg. KI	10	57 ± 2	7.7 ± 0.5	0.50 ± .13	68 ± 16	2.70 ± .17	26 ± 7	2.42 ± 0.34	312 ± 47	1.99 ± .10	159 ± 26
25 μg. KI	10	58 ± 1	6.4 ± 0.1	0.21 ± .04	33 ± 7	2.43 ± .17	14 ± 4	1.66 ± 0.12	266 ± 27	2.25 ± .18	123 ± 19

TABLE 5. THE EFFECTS OF 2 MG. KSCN, 6 MG. PROPYLTHIOURACIL, 20 MG. OF SULFADIAZINE SODIUM OR A CONTROL INJECTION OF DISTILLED WATER ON THE ONE HOUR THYROIDAL ACCUMULATION OF I^{131} OR Re^{188}. COMPOUNDS GIVEN IN A SINGLE SUBCUTANEOUS INJECTION ONE HOUR BEFORE ISOTOPE ADMINISTRATION. THIRTY-TWO-DAY OLD-MALE SPRAGUE-DAWLEY RATS MAINTAINED ON LOW-IODINE DIET FOR 7 DAYS. MEAN AND STANDARD ERROR REPORTED

Treatment	N	Body weight (gm.)	Thyroid weight (mg.)	Per cent of administered isotope found in sample							
				Re^{188}			Ratio	I^{131}			Ratio
				Thyroid gland	Thyroid per gm.	Plasma per cc.	Thyroid (gm.) / Plasma (cc.)	Thyroid gland	Thyroid per gm.	Plasma per cc.	Thyroid (gm.) / Plasma (cc.)
None	10	58 ± 1	8.3 ± .3	1.46 ± .28	167 ± 49	2.17 ± .38	77 ± 5	14.56 ± 1.82	1,709 ± 49	1.65 ± .43	1,321 ± 396
KSCN	10	56 ± 2	8.2 ± .3	0.21 ± .05	27 ± 6	3.37 ± .37	8 ± 2	3.69 ± 0.61	448 ± 24	2.58 ± .08	173 ± 30
Propylthiouracil	10	58 ± 1	8.0 ± .3	2.68 ± .18	332 ± 14	2.17 ± .12	154 ± 8	3.03 ± 0.16	381 ± 8	1.78 ± .08	217 ± 20
Sulfadiazine	10	59 ± 1	8.6 ± .4	2.76 ± .34	314 ± 29	2.61 ± .27	123 ± 14	2.95 ± 0.46	356 ± 50	1.88 ± .15	187 ± 20

TABLE 6. THE DISTRIBUTION OF Re^{188} OR I^{131} IN VARIOUS TISSUES AND ORGANS 20 HOURS AFTER RECEIVING THE ISOTOPE. FORTY-FIVE-DAY-OLD MALE SPRAGUE-DAWLEY RATS THAT WERE MAINTAINED ON THE LOW-IODINE DIET FOR TWENTY-TWO DAYS. MEAN AND STANDARD ERROR REPORTED FOR THYROID, BLOOD AND PLASMA, RANGE REPORTED FOR ALL OTHER VALUES

	N	Per cent of administered isotope			
		Re^{188}		I^{131}	
		% per gram	%/gram %/cc. plasma	% per gram	%/gram %/cc. plasma
Thyroid	10	10.6 ±4.4	94 ±15	6418 ±64	42426 ±6078
Blood	10	0.09 ± .01	—	0.11 ± .01	—
Plasma	10	0.10 ± .02	—	0.16 ± .02	—
Brain	4	0.002 – .006	0.03– .05	0.009– .014	0.08– .08
Pituitary	4	.001 – .008	.02– .04	.002– .016	.02– .09
Testis	4	.01 – .06	.22– .32	.01 – .37	.03– .33
Adrenal	4	.01 – .04	.08– .22	.04 – .06	.25– .54
Kidney	4	.10 – .30	.50– .75	.06 – .09	.49– .54
Liver	4	.09 – .22	.44– 5.55	.06 – .96	.52– .54
Spleen	4	.16 – .16	.78– 3.79	.04 – .06	.36– .36
Stomach	4	.50 – .82	4.10–12.37	.33 – .33	1.86– 3.00
Gut	4	.01 – .05	0.24– 0.25	.20 –1.10	1.10–10.00
Salivary	4	.01 – .06	.20– .32	.06 –0.24	0.32– 2.18
Sternum	4	.01 – .04	.22– .25	.03 – .03	.14– 0.24
Skin	4	.07 – .09	.35– 2.28	.50 – .80	.44– .45
Muscle	4	.01 – .02	.12– 0.15	.01 – .02	.06– .15
Lung	4	.02 – .13	.48– .67	.03 – .10	.27– .54
Heart	4	.01 – .06	.28– .30	.03 – .06	.27– .33

there were no great differences noted in the distribution of the two isotopes and, since Baumann *et al.* (1949a) has reported that Re^{186} was rapidly excreted as was I^{131}, no further discussion of these findings will be attempted.

DISCUSSION

It seems clearly established, by the previous work of Baumann *et al.* (1949a) and the data here reported, that the thyroid gland has the capacity to accumulate rhenium. However, there were certain differences noted between the thyroidal accumulation of I^{131} and Re^{188} that indicated that the thryoid gland handled the two elements differently. The most striking difference noted was the difference in magnitude of thyroidal accumulation of the two elements with the advantage in favor of I^{131}. An explanation for the difference between the shape and magnitude of the Re^{188} and I^{131} thyroidal accumulation curves was suggested by the observation that the I^{131} thyroidal accumulation curve of thiouracil treated animals was similar to the Re^{188} thyroidal accumulation curve. It has been demonstrated [Chaikoff and Taurog, 1949] that blocking thyroid hormone synthesis by thiouracil and studying I^{131} uptake in these blocked glands gave curves of the same type as the Re^{188} curve. The similarity of the Re^{188} and thiouracil blocked I^{131} curves suggested that Re^{188} was probably not organically bound. This inference was strengthened by the observation that no Re^{188} was present in the protein fraction of the gland. It is therefore, suggested

that the thyroid gland does not possess the capacity to convert Re^{188} to an organic form.

The finding that stable iodide lowered the thyroidal accumulation of I^{131} and Re^{188} is in agreement with similar studies done by Hamilton *et al.* (1953) where it was shown that iodide lowered the uptake of At^{211}, as well as I^{131}. The failure of stable rhenium to lower the thyroidal accumulation of I^{131} agrees with the majority of evidence which suggests that flourine, chlorine and bromine fail to depress the uptake or retention of I^{131} as iodide [Wyngaarden *et al.*, 1952] although Baumann [1953] holds that rhenium depresses I^{131} uptake. The failure of stable rhenium to lower the accumulation of Re^{188}, as noted in the present study, is even more puzzling and does not agree with the data presented by Baumann [1949a] which showed that 100 μg. of rhenium had a depressing effect upon the uptake of Re^{186}. It is felt that the differences in experimental design and the small sample size utilized in both experiments make the results on the ability of rhenium to depress thyroid function, in regard to the accumulation of halogens, inconclusive and subject to further investigation.

There seems to be general agreement that SCN^-, in some fashion, lowers the accumulation of iodide. The demonstration that KSCN lowered the thyroidal accumulation of At^{211} was taken as evidence that the thyroid gland accumulated astatide [Shellabarger *et al.*, 1954]. It seems reasonable to assume that the Re^{188} that was accumulated by the gland was also accumulated as an anion.

It was of some interest to examine the magnitude of iodide-trapping ability of the thyroid gland for I^{131} and to compare this value with the amount of Re^{188} accumulated by the gland. These two values might be expected to be of the same general magnitude if the assumption that Re^{188} was not organically bound is valid, since it has been demonstrated that iodide can be initially fixed in the thyroid by a mechanism that is distinct from the reactions responsible for converting it to an organic form [Schachner *et al.*, 1944]. When the I^{131} uptake of the thiouracil blocked glands was compared with the Re^{188} values at one hour after isotope administration, the percentages were not dissimilar: 3.5 to 2.2 and 3.7 to 2.6, I^{131} and Re^{188} respectively. It would seem likely that the mechanism that enables the thyroid to accumulate iodide does not readily distinguish iodide from Re^{188}.

The curious reports [Durbin *et al.*, 1954, Shellabarger and Godwin, 1954] that thiouracil or propylthiouracil either failed to lower or increased the thyroidal accumulation of At^{211} have not been completely understood. The findings in the present study are similar to the above reports, i.e., the administration of thiouracil or propylthiouracil either failed to lower or increased the thyroidal accumulation of Re^{188}. In the case of the At^{211} studies, the suggestion was advanced [Durbin *et al.*, 1954] that the thiouracil type compounds actually increased the organic binding of At^{211} which in turn, increased the thyroid content of the isotope. It is felt that an alternative explanation can be offered that is equally tenable, and that this explanation will also serve as a model to explain the enhanced Re^{188}

accumulation produced by the thiouracils that was noted in the present study. It has been shown subsequently that the KSCN discharged approximately fifty per cent of the At^{211} held by the thyroid [Shellabarger *et al.*, 1954]. This finding was taken to mean that astatine was not bound in a fashion strictly comparable to the organic binding of I^{131}. The demonstration that the thiouracils increase the ability of the thyroid gland to trap iodide [VanderLaan and VanderLaan, 1947; Halmi *et al.*, 1953; VanderLaan and Caplan, 1954; Vanderlaan, 1954] might well be extended to At^{211} and Re^{188}. Although the mechanism of an increased capacity of thiouracil-treated gland to trap iodide is imperfectly understood, it seems reasonable to assume that the enhanced capacity of the thyroid gland to accumulate At^{211} or Re^{188} under the influence of certain goitrogens may likewise be a reflection of the enhanced capacity of the gland to trap elements in the minus one valence state. It should be remembered that, although the thyroid gland has the capacity to accumulate Br^{82}, no organic binding of bromine has been demonstrated [Yagi *et al.*, 1953]. All of the evidence concerning thyroid function and elements other than iodine seem to suggest that, although the gland has the capacity to accumulate these elements, there have been no direct demonstrations that the thyroid gland has the capacity to bind these elements in an organic form.

The chemistry of rhenium would suggest that the Re^{188} remained in the form of an anion ReO_4^- [Sidwick, 1954]. If this is true, the demonstration that the thyroid gland selectively accumulated ReO_4^- is of interest, since it has been demonstrated that the SCN^- ion was not selectively accumulated by the gland [Wood & Williams 1949] and that propylthiouracil did not change the amount of SCN^- found in the gland [Wood and Kingsland, 1950]. In view of the extreme anti-thyroid action of perchlorate on the ability of the gland to trap and retain iodide [Wyngaarden *et al.*, 1952, Wyngaarden *et al.*, [1953], it would seem of interest to ascertain if the perchlorate ion is accumulated by the thyroid and to examine the possibility that perrhenate might also prove to be an anti-thyroid compound.

SUMMARY

Re^{188} was found to be selectively accumulated by the rat thyroid gland, but to a lesser extent than I^{131}. The shape and magnitude of the Re^{188} accumulation curve resembled the shape and magnitude of thiouracil blocked I^{131} curve. This was interpretated as evidence that Re^{188} was not organically bound, and this was confirmed by the observation that no Re^{188} was found in the portein fraction of the thyroid gland. KSCN lowered the thyroidal accumulation of Re^{188} and this was thought to mean that the thyroid gland trapped Re^{188} as an anion. The magnitude of the iodide trap was approximately the same as the amount of Re^{188} found in the gland. It was suggested that thiouracil enhanced the trap of Re^{188} and that this was sufficient to explain the increased thyroidal accumulation of Re^{188} that was observed with goitrogen treatment.

The reports that the thyroid gland has the capacity to accumulate ele-

ments other than iodine were discussed and it was concluded that the evidence to date suggests that, although the thyroid gland may possess the capacity to accumulate elements other than iodine, it seems likely that the thyroid gland does not bind these elements in a fashion comparable to the organic binding of iodine.

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THE MECHANISM OF THE ANTITHYROID ACTION OF IODIDE ION*

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THE mechanism whereby iodide exerts its antithyroid action has been the subject of speculation from the time that its beneficial effect on Grave's disease was first noted. The phenomenon has been brought under experimental control and it is known that sufficient concentrations of iodide will completely block the formation of organically-bound iodine both in normal thyroid tissue and in surviving thyroid slices (Wolff and Chaikoff 1948; Morton, Chaikoff and Rosenfeld 1944).

Two of the various theories advanced to explain this action of excess iodide are based on well-known chemical reactions of iodide ion. The first of these, which was advanced by Pitt-Rivers (1950) and Harington (1951), postulates that hypoiodous acid is the reactive form of iodine which enters organic combination in the gland. This substance is formed by the hydrolysis of elemental iodine:

$$H_2O + I_2 \rightleftarrows HIO + H^+ + I^-. \qquad [1]$$

It is obvious that the addition of iodide ion to this system will decrease the concentration of hypoiodous acid. The second theory, advanced by Fawcett and Kirkwood (1953), states that iodide acts through its ability to combine with elemental iodine to form a molecular compound:

$$I^- + I_2 \rightleftarrows I_3^-. \qquad [2]$$

This mechanism will result in the inhibition of the iodination process in the gland regardless of the nature of the reactive species involved. Before iodide can enter an aromatic substitution reaction, it has to be oxidized to the level of elemental iodine and it is either elemental iodine, or some positive species in equilibrium with it, which is involved in all aromatic substitution reactions involving this element.

These two theories are bolstered by the demonstration that elemental iodine is formed in the thyroid gland and is the first intermediate in the process of the organic binding of iodine in the thyroid (Fawcett and Kirkwood 1953). These mechanisms have the merit that they will not only explain this *in vivo* action of iodide ion, but will also account for the fact that iodide inhibits the general reaction between iodine and aromatic sub-

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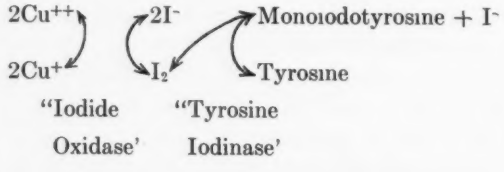
stances (Li 1942; Berliner 1950). Both mechanisms are equally probable on theoretical grounds, and which, if either, actually operates in the gland will depend on the nature of the iodination processes utilized by the thyroid. A decision between the two could be made if quantitative data were available on the extent of inhibition produced by various concentrations of iodide. Since the equilibrium constants of both reactions [1] and [2] are known, it is possible to predict with considerable accuracy how they would affect any iodine-utilizing system. It is difficult to obtain such data from a study of the action of iodide on the thyroid gland *in situ*; however, such an attempt has recently been made (Wollman and Scow 1954).

Since iodide in sufficient concentration will totally prevent the formation of organically-bound iodine in the thyroid, it must necessarily inhibit the first step in the organic binding process. Fawcett and Kirkwood (1953, 1954) have recently shown that is it possible to extract from thyroid and salivary gland tissue a soluble enzyme which, when supplemented with cupric ion and tyrosine, catalyzes the formation of monoiodotyrosine (MIT), the first intermediate in the synthesis of thyroxine. These authors suggested (1953) that the function of cupric ion was to accept electrons directly from iodide ion, thus replacing the "iodide oxidase" which is responsible for the formation of elemental iodine in the intact thyroid gland. Recent evidence indicates that this view will have to be modified. A study of the effect of iodide concentration on the rate of iodination of tyrosine, and a study of the iodination of extremely reactive iodine acceptors by this system, indicate that iodine is being produced by an enzyme system (Serif and Kirkwood, 1955). This enzyme is presumably the iodide oxidase which functions in intact thyroid and salivary gland tissue. It catalyzes an equilibrium between electron donors or acceptors and the iodide-iodine system. Thus it will catalyze either the oxidation of iodide, or the reduction of iodine, depending on the redox potential of the system. Its function in the copper supplemented system can be represented by:



Since this enzyme system can be prepared iodide-free, it forms a suitable "model" with which to make a quantitative study of iodide inhibition. A direct correlation can also be made between the function of the isolated enzyme system and the function of the organic binding process in the intact thyroid gland (Wollman and Scow 1954), and should provide evidence as to whether or not this enzyme functions in thyroid tissue.

EXPERIMENTAL

Homogenate Techniques.—The dialyzed material used in these experiments was prepared from 20% homogenates of rat submaxillary gland tissue.[1] The tissue was homogenized, in ice-cold isotonic KCl, in a Potter-Elvehjem glass homogenizer maintained at 0° in an ice-bath. The homogenates were centrifuged at 0° for 20 minutes at 2500×g. to remove stroma and unbroken cells. The supernatant solution was then placed in cellophane dialysis tubes and dialyzed, with stirring, for 40 hrs. against 2 changes of 0.1 M phosphate buffer (4 liters per change), pH 7.4, prepared from salts purified as described below. The dialyzed material was then centrifuged for 20 minutes at 0° and 2500×g. to remove some precipitated material. This supernatant solution, which was made up to the same volume as the original homogenate with 0.1 M phosphate buffer, was used as the enzyme solution in all experiments.

Experiments were carried out in 125×15 mm. pyrex test tubes containing the following mixture: 1.0 ml. of the appropriate homogenate, 0.5 ml. of 0.1 M phosphate buffer, pH 7.4, prepared from salts purified as described below, 0.2 ml. of 0.04 M $CuCl_2$, 0.2 ml. of 0.04 M tyrosine, and 1.4 ml. of the appropriate concentration of KI and radioiodide. The labelled iodide was carrier-free and was used in different amounts, depending on the level of iodide in the medium. In experiments containing iodide concentrations of from 10^{-6} to 10^{-5} M KI, 20 μc. of activity was used. In those containing from 10^{-5} to 10^{-3} M KI, iodide of constant specific activity was used such that the tube containing 10^{-3} M iodide contained 0.75 mc. of activity. By doing parallel experiments with material of one-tenth this specific activity it was shown that the radiation was not affecting the performance of the enzyme system. The high levels of radioactivity were used in order to enable the amount of MIT formed at the higher iodide concentration to be measured with the requisite precision. As the concentration of stable iodide is increased, the percentage incorporation of radioiodine into MIT is markedly reduced due to the lowering of the specific activity of the administered iodide.

Methods of Paper Chromatography and Radioautography.—All preparations were chromatographed on Whatman No. 1 paper which had been pre-treated with buffer. The general techniques used have been described elsewhere (Fawcett and Kirkwood 1953, 1954). All samples were dried on the paper with 3 μg. of sodium thiosulfate. This technique greatly improves the quantitative estimation of radioactivity by the method described below. More recently Taurog *et al.* (1955) have advocated the use of thiouracil for the same purpose. The position of the radioactive spots was determined by radioautography (Fawcett and Kirkwood 1953).

Quantitative Estimation of Radioactivity Present in Chromatographic Spots.—In order to determine the proportion of iodine in the different chemical forms present at the end of each experiment, the corresponding spots on the chromatograms were cut out, folded into small squares (approximately 5×5 mm.) and counted with a scintillation counter. The radioiodine present as MIT was calculated as a percentage of the total radioiodine present on the chromatogram. This automatically corrects for decay of the activity and also corrects any small errors which may have occurred in measuring the aliquot dried on the paper.

Purification of Salts Used in Dialysis.—In order to obtain tissue homogenates that were free of iodide it was necessary to dialyze against buffer prepared from carefully purified

[1] Rat submaxillary tissue was chosen as a source of enzyme in the present work because the small size of the rat thyroid prohibits preparation of workable amounts of homogenate. Insofar as we have been able to determine the enzyme in the submaxillary gland is identical, in specificity and behaviour, to that found in the thyroid.

salts. The salts ($Na_2HPO_4 \cdot 7H_2O$ and KH_2PO_4) were purified by dissolving the analytical reagent grade material in approximately twice the volume of distilled water necessary to make a hot, saturated solution. Hydrogen peroxide (3.0 ml. of 30%) was added to the solution and it was then evaporated to one-half its volume. The solution was allowed to cool, and the precipitated salt filtered off on a Buchner funnel. This procedure was repeated twice more.

RESULTS AND DISCUSSION

Influence of Iodide Concentration on the Rate of Synthesis of Monoiodotyrosine by Homogenate Preparations.—The effect of iodide on the rate of synthesis of MIT by dialyzed preparations of rat submaxillary gland is shown in Figure 1. A typical substrate saturation curve would be predicted for this plot since the rate of formation of MIT should be limited either by the saturation of "iodide oxidase" with iodide ion or by the saturation of "tyrosine iodinase" with elemental iodine produced by the iodide oxidase enzyme. Either of these mechanisms would produce a curve that increases with iodide concentration and then approaches a limiting velocity. It is seen from Figure 1 that the velocity increases to a maximum value and then falls as the iodide concentration is further increased. Thus this enzyme system is inhibited by concentrations of iodide above the optimum value just as is the process of organic binding in the thyroid gland. The curve could not be extended much beyond iodide concentrations of 10^{-3} M. The amount of MIT synthesized in a given time was determined by multiplying the fraction of radioiodide converted to MIT by the molar concentration of iodide present in the medium. Since the fraction of radioiodide converted to MIT became increasingly lower as the iodide concentration was raised, both owing to inhibition and to dilution of the specific activity of the iodide, the accuracy of its measurement steadily decreased. Although this was partially compensated for by increasing the amounts of radioiodide used, 10^{-3} M iodide represents the highest iodide concentration at which reliable values could be obtained.

The Mechanism of the Inhibition.—The possibility that the enzyme-system is being inhibited by either reaction [1] or [2] can be investigated by calculating the extent of inhibition to be expected from each reaction and comparing it to the observed inhibition.

The equilibrium constant of reaction [2] is known and has the value (Davies and Gwynne 1952):

$$K = \frac{(I_3^-)}{(I^-)(I_2)} = 590 \text{ at } 38°. \qquad [3]$$

This can be rearranged to:

$$\frac{(I_2)}{(I_3^-)} = \frac{0.00170}{(I^-)}. \qquad [4]$$

Expression [4] allows us to calculate the extent of inhibition produced by any concentration of iodide ion, provided that the mechanism of inhibition

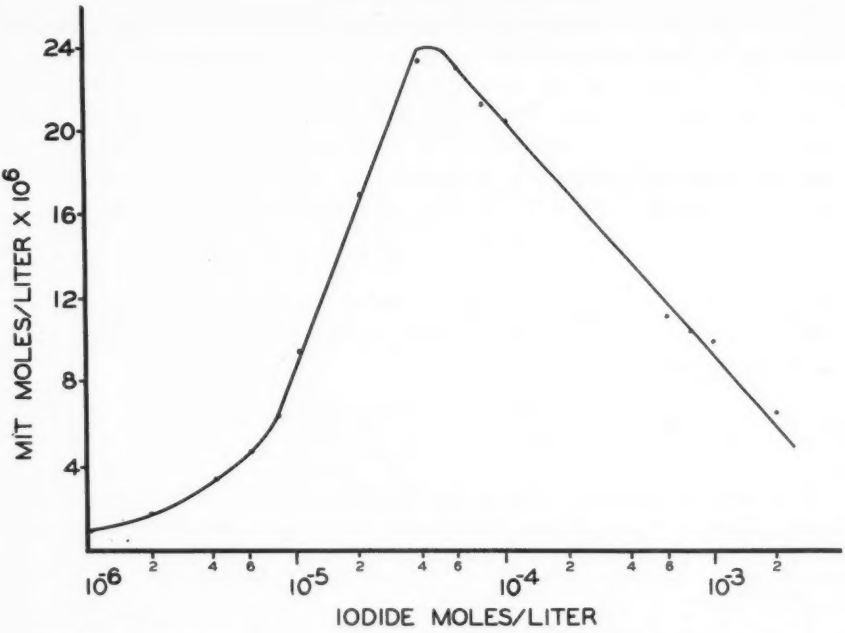


Fig. 1. Amount of monoiodotyrosine (MIT) synthesized in three hours by dialyzed enzyme preparations, as a function of iodide concentration.

is through reaction [2]. This follows since $(I_2)/(I_3^-)$ is a measure of the iodinating species in bound form, and the steady state concentration of elemental iodine is constant at iodide concentrations above the optimum.

A calculation of this ratio at the optimum iodide concentration (5×10^{-5} M) shows it to be in accord with the experimental fact.

$$\frac{(I_2)}{(I_3^-)} = \frac{0.00170}{5 \times 10^{-5}} = 34.$$

Under these conditions only about 3% of the iodine is bound in the form of the complex. The value of this ratio at the level of iodide producing 55% inhibition (10^{-3} M) is 1.7, that is, 35% of the available iodine is bound in the inactive form. It is obvious from this that the major part of the inhibition can be accounted for on the basis that is it mediated through reaction [2].[2]

[2] It should be pointed out that the apparent equilibrium constant of reaction [2] increases if the concentration of elemental iodine is increased above a certain minimum value (Davies and Gwynne, 1952). This is caused by further associative reactions between iodide and iodine which only come into prominence of higher iodine concentrations. Should there be a sufficient concentration of elemental iodine, or any species in equilibrium with it, upon an enzyme surface, then these further reactions would take place. This would cause the calculated inhibition to be lower than that actually observed.

The inhibition to be expected from reaction [1] can be calculated in the following fashion. The equilibrium constant for this reaction is:

$$K = \frac{(HIO)(H^+)(I^-)}{(I_2)(H_2O)}. \qquad [5]$$

This can be rearranged to:

$$(HIO) = \frac{K(I_2)(H_2O)}{(H^+)(I^-)}. \qquad [6]$$

It is possible to calculate the ratio of HIO concentration at the optimum iodide concentration to the HIO concentration at 55% inhibition by the use of this expression thus:

$$\frac{(HIO)_{opt}}{(HIO)_{inhib}} = \frac{(I_2)_{opt}(I^-)_{inhib}}{(I_2)_{inhib}(I^-)_{opt}}. \qquad [7]$$

It is possible to calculate $(I_2)_{inhib}$ as a fraction of $(I_2)_{opt}$ from equation [4], which yields a value $(I_2)_{inhib} = 0.65(I_2)_{opt}$. When this and the appropriate iodide values are substituted in equation [7] the value for the ratio is:

$$\frac{(HIO)_{opt}}{(HIO)_{inhib}} = 31.$$

Since the velocity of the reaction will be directly proportional to the HIO concentration, if it is the iodinating species, an inhibition of 97% would be expected. It is obvious that this is considerably more than the observed inhibition and, in the authors' opinion, rules out reaction [1] as a mechanism for the observed inhibition.

Correlation of the Inhibition Observed In Vivo with that Observed with the Enzyme Preparation.—Recently Wollman and Scow (1954) have attempted to decide between the Fawcett and Kirkwood mechanism and the Pitt-Rivers and Harington mechanism on the basis of the inhibition of organic binding of radioiodine observed in intact mouse thyroid, after the injection of graded doses of iodide. They come to the conclusion that the Fawcett-Kirkwood mechanism will account reasonably well for the inhibition produced by higher levels of iodide, but will not account for the inhibition first observed as the level of iodide in the gland is increased. They make the erroneous assumption that a decrease in the amount of radioiodide bound by the gland is a direct measure of inhibition of the binding process. It should be pointed out that this is not the case and that the addition of stable iodide to any iodinating system will lower the amount of radioiodide bound merely by decreasing its specific activity, while at the same time it may not affect, in any way, the amount of organic-iodine formed. This is seen if both the per cent radioiodide bound in MIT, and the actual number of moles of MIT synthesized, are plotted against added

iodide, using the dialyzed enzyme system previously described (Fig. 2). It is evident that the per cent incorporation of radioiodide falls off at iodide levels considerably below those that produce the maximum rate of synthesis of MIT. An examination of Wollman and Scow's data on the actual amount of iodine bound in μg./hour shows that they observe no inhibition in organic binding at iodide concentrations where the Fawcett-Kirkwood mechanism predicts there should be none and that the amount observed at

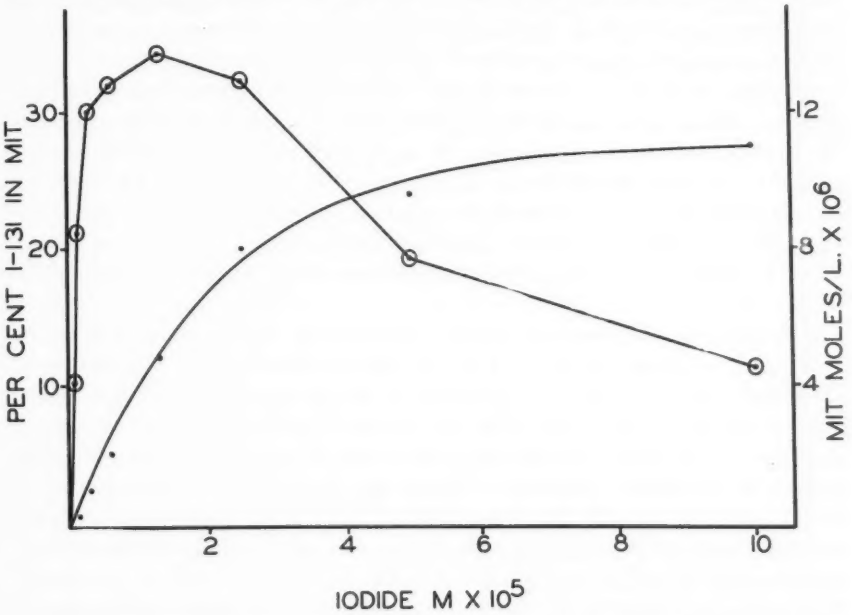


FIG. 2. Per cent incorporation of radioiodine into monoiodotyrosine (MIT) as contrasted with the amount of MIT actually synthesized. Per cent incorporation I-131 into MIT (left ordinate, circled points). MIT synthesized, in moles per liter (right ordinate).

higher iodide concentrations agrees quite well with that predicted by this theory.

The Active Iodinating Species in the Gland.—The identification of the active species in the iodination of aromatic substances in aqueous solution has been the subject of considerable investigation (Ingold 1953). There can be little doubt that the reactive species is an electrophilic agent and probably is either positive iodide ion (I^+, iodinium ion) or the hydrated form of this ion, IOH_2^+. The former seems to be favoured by the recent evidence (Berliner 1950, Ingold 1953). Indeed, there is no evidence as yet that any iodine carrier, other than the above cationic species, is effective for aromatic iodination in aqueous solution. It would not be expected, then, that HIO would be the active iodinating species in the enzyme-catalyzed

iodination of tyrosine, and the present work confirms this expectation. Neither would it be expected that fluctuations in the concentration of HIO would affect the concentration of the above-mentioned cations. Elemental iodine is a more effective source of I^+ than is HIO (Berliner 1950) and the equilibrium of reaction [1] is very far to the left, which prevents this reaction from appreciably influencing the concentration of elemental iodine.

The position with regard to elemental iodine is quite different. It is in equilibrium with both I^+ and the hydrated form of this ion, and any agent that decreases the concentration of elemental iodine will also decrease the concentration of both of these cations. As has been pointed out, iodide ion causes marked decrease in the concentration of elemental iodine because the equilibrium point of reaction [2] is to the right (Davies and Gwynne 1952). It has been conclusively demonstrated that the inhibiting action of iodide on the iodination of aniline is mediated through its ability to form a complex with molecular iodine (Berliner 1950). There is no obvious reason why the enzyme-catalyzed iodination of tyrosine should behave in a different fashion.

The position of elemental iodine with respect to the active iodinating species in the gland cannot, as yet, be settled with any degree of certainty. It would appear that two possibilities are open. Iodide oxidase must remove two electrons from iodide ion to produce I^+. This may be passed directly to tyrosine iodinase, in which case I^+ is the active iodinating species in the gland. However, if this is the case, the I^+ on the surface of iodide oxidase is available to react with substances other than tyrosine iodinase since it is taken up by resorcinol and other reactive aromatic compounds, and therefore, it will also react with iodide to form elemental iodine (Fawcett and Kirkwood, 1953; Serif and Kirkwood 1955). This reaction pathway is shown in sequence [8]:

$$\begin{array}{c} I_2 \\ \uparrow\downarrow \\ I^- \rightleftarrows I^+ \leftrightarrows \text{MIT.} \\ \begin{array}{ll} \text{iodide} & \text{tyrosine} \\ \text{oxidase} & \text{iodinase} \end{array} \end{array} \qquad [8]$$

It is equally possible that elemental iodine is the product of iodide oxidase and that it is released to the environment where it is taken up by tyrosine iodinase. If this is the case, iodine is directly in the reaction pathway which is represented by [9]:

$$\begin{array}{c} 2I^- \leftrightarrows I_2 \leftrightarrows \text{MIT} \\ \begin{array}{ll} \text{iodide} & \text{tyrosine} \\ \text{oxidase} & \text{iodinase} \end{array} \end{array} \qquad [9]$$

If this is the reaction sequence, then tyrosine iodinase could catalyze the iodination of tyrosine by causing the elemental iodine to dissociate to I^+ on its surface, as well as dissociating the phenolic hydroxyl of tyrosine. Both of these events would greatly augment the rate of reaction.

Both reactions [8] and [9] are equally susceptible to inhibition by agents which combine with elemental iodine, and it will require a great deal of work with purified enzyme systems to determine which sequence is used by the thyroid gland.

Correlation with Clinical Observations on the Action of Iodide on the Thyroid.—If iodide exerts its action, in whole or in part, through the mechanism advanced here, then it would be predicted that the prolonged administration of iodide in sufficient quantities should cause the appearance of hypothyroidism and goiter in normal subjects. Turner and Howard (1955) have recently reported twelve cases of myxedema and simple goiter resulting from the prolonged ingestion of iodide-containing mixtures by normal subjects. The thyroid was reduced to normal and the hypothyroidism cured either by stopping the iodide or administering thyroxine. They review eight similar cases previously reported in the literature.

The mechanism advanced here also serves as a possible explanation for the action of iodide on Graves' disease. It has been drawn to the authors' attentions (Personal Communication from Dr. J. E. Rall) that iodide has a sudden and dramatic effect on the symptoms of Graves' disease that is not shared by even the powerful thiourea-type antithyroids. It is not possible to explain this sudden action on the basis of the mechanism advanced above, which would predict that iodide would have a weaker effect than the thiourea-type antithyroids. While it cannot be denied that iodide in sufficient quantity will completely block the synthesis of organically-bound iodine in the animal and human thyroid, it is entirely possible that iodide has other actions on the gland. Halmi and Spirtos (1955) have shown that the response of the gland to thyrotrophin is controlled by iodide intake. Thus there can be no doubt that iodide level controls at least one aspect of the action of thyrotrophin on the gland and it may well be an action of this type, coupled with the well-established inhibition of iodination, which accounts for the peculiar effect of iodide on Graves' disease.

ACKNOWLEDGMENT

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ADDENDUM

It has recently come to our attention that S. Wollman (Brookhaven Symposia in Biology No. 7, The Thyroid, p. 72, 1954) has concluded that the Fawcett-Kirkwood mechanism fails to account for the antithyroid ac-

tion of dioxane, since calculations based on the equilibrium constant for the dioxane-iodine interaction in hexane solution (Ketelaar *et al.*, Rec. trav. chim., *71*, 1104 (1952).) show that only a negligible fraction of the iodine would be complexed at a dioxane concentration that strongly inhibits organic binding in thyroid slices. It should be pointed out that there is no obvious reason to believe that the equilibrium constant of the dioxane-iodine interaction is the same in both hexane and aqueous solution. Indeed it has been shown that the solvent can have a profound effect on the equilibrium constant of the formation of molecular compounds (Ross and Kuntz, J. Am. Chem. Soc., *76*, 3000 (1954)).

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A QUANTITATIVE METHOD FOR MEASURING MELANOPHORE EXPANDING ACTIVITY[1]

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INTRODUCTION

A NUMBER of methods have been developed recently for the estimation of melanophore expanding activity in pituitary extracts. The majority of them are carried out *in vitro* utilizing isolated frog skin. There is, however, a need for a quantitative method of estimating melanophore expanding activity *in vivo* since there are indications that the melanophore responses *in vivo* are more specific for the melanophore expanding principle, and not influenced by many other substances that may be present in biological extracts (Sulman 1952a) (Schizume 1954).

The available *in vivo* methods utilize the degree of melanin dispersion within the melanophores (melanophore index) estimated by microscopic observation as a measure of the response. It was felt that an objective measurement of the response should be used to obtain results that can be treated statistically and thus permit an evaluation of the accuracy and precision of the assay.

The object of the experiments reported here was to study the characteristics of melanophore responses of intact animals (frogs) when measured by photoelectric means. Quantitative data thus obtained were analyzed statistically to determine the limitations and reliability of the method, and to serve as a basis for an *in vivo* bioassay.

MATERIALS AND METHODS

The method consists of a series of photoelectric measurements of the light reflected from the dorsal surface of intact frogs, before and at various time intervals after the injection of a pituitary preparation. Photoelectric measurements were made with a Photovolt Densitometer (model 501A).[2] A cylindrical brass tube, with a side-arm, was attached to the face of the photocell container. This tube was 7.5 cm. in height and had an internal diameter of 2 cm. A small flashlight bulb was secured into the side arm so as to project light onto the dorsal surface of the frog without permitting light to pass directly from the bulb to the photocell. The intensity of the light projected was kept constant by means of a galvanometer and two variable resistors. By adjusting the resistors a reading on the

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[1] This investigation was supported by a grant from Parke, Davis and Company, Detroit, Michigan.

[2] Photovolt Corporation, New York, New York.

galvanometer could be maintained constant during the period that the bulb was illuminated, insuring a constant intensity of light throughout the period that readings were made. The line voltage to the light source, obtained through a transformer, was stabilized by means of a constant voltage regulator. Furthermore, the light reflectance from a white enamel surface was measured at frequent intervals to assure constancy of the recording system.

Readings were taken after pressing the cylinder tightly on the dorsal surface of the frog and maintaining it there by a clamp attached to a burette stand. By this means the total amount of light reflected from the frog's skin was measured without loss. The area on which the first reading was taken was recorded, so that the same area could be measured in subsequent readings. This circular area was approximately 3.14 cm.^{2}.

Intact summer frogs (Rana pipiens) were previously light-adapted by placing them, in groups of six, in white enamel pans covered with wire mesh screening which were under continuous illumination from a 200 watt light bulb, eighteen inches above. Animals were considered ready for use when they showed at least 19% reflectance at the scale #2 of the densitometer. These animals were very light green-yellow to the naked eye, corresponding to (−) in Sulman's scale (1952b). Under our conditions three to six weeks of continuous exposure to light was required for the animals to reach this stage of light adaption.

The animals were restrained in a prone position by clips on boards covered with sheets of white paper. During the experiment, except for the time a reflectance measurement was made, the preparations were covered with moist cheese-cloth in order to keep their skin moist and permit respiration.

As a source of melanophore expanding principle an ACTH preparation was used, since such preparations are known to contain large quantities of this principle (Sulman, 1952a; Johnson and Hogberg, 1952). The ACTH used was an Armour preparation, Lot No. L 60311. The vials were labelled to contain 25 I.U. of ACTH. We found that each vial contained 16 mg. of dry powder. Its potency was, therefore, calculated as 1.56 I.U. of ACTH per mg. In the present study all doses are expressed as micrograms of dry powder. For the determination of the standard curve, solutions were made with frog Ringer's (pH 7.4 to 7.6) and injected into the dorsal lymph sac, in doses ranging from 0.0064 to 64 μg./frog. Each experimental group consisted of 10 frogs, each frog receiving a dose of ACTH in a volume of 0.1 cc. In addition a total number of 18 control frogs received 0.1 cc. of Ringer's solution. Injections were made five minutes after an initial reading was taken. Following the injections, readings were taken every 15 minutes for a period of 90 minutes.

In order to determine whether the responses of the frogs varied with the season of the year, determinations of the melanophore expanding activity of the same ACTH preparation were made utilizing two dose levels and 5 to 15 animals per dose group. In these experiments the light reflectance of the animals was measured just before and one hour after injection.

RESULTS

Standard tests of normality were applied to the values of the amount of light reflected from each frog before injection. A Chi-Square test, following the method of Gaddum (1945), yielded a value corresponding to a probability of greater than 99% that the distribution is normal. In addition a g-test of symmetry (Snedecor, 1946) gave a t-value of −0.35, which is distinctly within experimental error. It is, therefore, concluded that the values are normally distributed and that there is no significant deviation

from symmetry in the distribution of these values around their mean. This justifies the use of the analysis of variance and F-tests which imply a normal distribution. Analysis of variance showed no significant difference among the means of the experimental groups ($F = 1.49$), which indicates that the distribution of the animals among these groups was homogeneous in this respect.

Light reflected from the control groups showed no statistically significant changes throughout the experimental period ($F = 1.1$). Mean reflectance of the control and each experimental group was computed at each time interval after injection, and time-response curves were plotted for each dose (Fig. 1). Reflectance values, plotted on the ordinate, were also

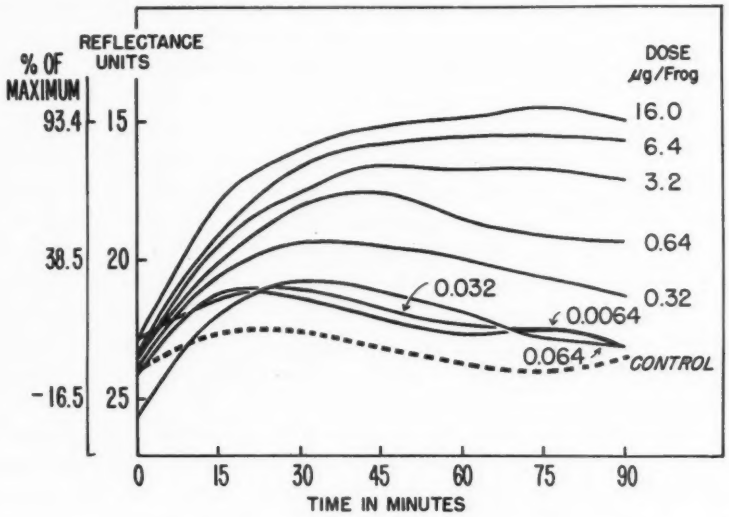

FIG. 1. Time-response curves for experimental and control frogs throughout the period of observation.

The curves for 32 and 64 µg./frog were identical with the curve for 16 µg./frog.

computed and expressed as per cent of maximum response. For this, 14.5 is taken as mean-maximum response and 23.5 as mean starting value, giving a maximum difference of 9.0. The difference between the mean response at each time interval and 23.5 is then expressed as per cent of the maximum difference (i.e., 9.0).

The results of the minimum reflectance during the experimental period corresponding to the maximal darkening of the frog to a given dose (peak-response), and those of the reflectance at one hour after injection (one-hour-response) were analyzed.

The responses to a given dose expressed either as peak-response or one-hour-response showed considerable more variation when calculated as per

cent change (taking into consideration the initial reflectance of the frog before injection), than when the actual values were considered. It was desirable, however, to find out whether there was any dependence of the response on the reflectance before injection. Covariance analysis (Emmens 1948) showed that there was a small but significant dependence of the peak and one-hour-responses on the reflectance before injection. Correcting for covariance reduced the variance of error to about 65% of its previous value, i.e. it increased the precision by about 35%.

Means and standard deviations of the peak and one-hour responses for each dose group were corrected by covariance analysis and are recorded in Table 1.

Covariance analysis showed that the peak-response and one-hour-re-

TABLE 1. PEAK AND ONE-HOUR RESPONSES (REFLECTANCE UNITS) BEFORE AND AFTER INJECTION

	Dose μg./frog										
	Control	0.0064	0.032	0.064	0.32	0.64	3.2	6.4	16	32	64
Before Injection											
Mean	23.8	23.8	22.8	25.7	23.9	23.5	22.9	23.9	22.8	23.0	23.1
±S.D.	2.6	3.0	2.4	2.9	2.9	2.7	2.6	2.4	2.1	2.6	3.5
After Injection*											
Peak-response:											
Mean	21.9	20.8	20.9	19.0	18.7	17.5	16.0	15.1	14.6	14.5	14.3
±S.D.	2.1	1.5	1.5	1.2	1.5	1.3	0.9	1.5	2.4	1.0	1.5
One-hour-response:											
Mean	23.6	22.5	22.5	21.0	19.7	18.6	17.0	15.4	15.3	15.0	15.2
±S.D.	2.0	1.5	1.4	2.0	1.8	1.7	1.8	1.6	2.3	1.3	1.8

* Corrected for initial reflectance by covariance analysis.

sponse were *independent* of the animal's weight. The weights of the frogs used in these experiments were within a range of 14–45 gm., with mean and S.D. of 28.4 ± 7.0 gm. They were distributed at random among the different dose groups.

Peak and one-hour-responses to 0.0064 and 0.032 μg./frog did not differ significantly from the control group, whereas the responses to 0.064 μg./frog did. Responses to 16, 32 and 64 μg./frog were found not to be significantly different. We, therefore, took the mean response to these doses to indicate the maximum response under these conditions.

Dose-response regression lines for the peak-response and one-hour-response were computed between the doses of 0.064 and 6.4 μg./frog inclusive. The equation of the line for the peak-response was found to be: $\overline{Y} = 17.0 - 2.04 \log x$ and for the one-hour-response: $\overline{Y} = 18.1 - 2.73 \log x$. When expressed as per cent of the maximum change (as described for the time-response curves) the equations of the lines become: for the peak-

TABLE 2. COMPARISON BETWEEN PEAK-RESPONSE AND ONE-HOUR-RESPONSE

	Slope	Variance of error	Precision index
	b	Ve	λ
Peak-response	2.04	0.83	0.45
One-hour-response	2.73	1.97	0.51

response: $\overline{Y} = 72.2 + 22.7 \log x$ and for the one-hour-response: $\overline{Y} = 60.0 + 32.9 \log x$.

Analysis of variance of the log dose-response regression lines showed the regressions to be highly significant and the computed straight lines to fit the data well. Bartlett's Chi-Square test (Emmens, 1948) was applied to the variances (corrected for covariance) of the dose groups of 0.064 to 6.4 μg./frog inclusive. It indicated homogeneity among the variances.

Theoretically the best measure of melanophore response is the maximum or peak-response to a given dose (Landgrebe and Waring 1950). Hence, no matter what the ultimate choice of response may be for practical reasons, it must be compared to the theoretically best measure (i.e., to the peak-response). A comparison was made between the does-response regression line of the peak-response versus the regression line of the one-hour-response. This comparison showed that the variance of error of the one-hour-response is significantly greater than the variance of error of the peak-response, while the slope of the regression line of the peak-response is smaller than that of the one-hour-response (Table 2). Thus the precision indices (Gaddum 1933) which are an indication of the over-all accuracy of each dose response regression line, are not significantly different. Therefore, the peak-response would be expected to give no more accurate results than the response measured at one hour following injection. This conclusion may not hold true when the time-response curve is considerably altered.

Six log dose-response curves of the standard Armour ACTH preparation were obtained between the months of March and November and were

TABLE 3. CHARACTERISTICS OF DOSE-RESPONSE LINES OF STANDARD ACTH PREPARATION (MARCH-NOVEMBER 1954)

Month	No. of animals	Slope	Variation	Precision index
		b	SD	
March	14	2.7	1.4	0.51
April	10	3.5	1.6	0.44
May	30	4.5	1.5	0.34
May	10	6.5	2.1	0.32
May	10	3.7	1.2	0.33
August	20	3.3	1.4	0.42
November	6	4.7	1.8	0.38
Total	100			
Mean ±SD		4.1 ± 1.3	1.6 ± 0.3	0.39 ± 0.07

compared with respect to slope, standard deviation, and degree of precision (lambda, λ). The results are presented in Table 3.

DISCUSSION

The response of frogs to a melanophore expanding hormone preparation shows great variability from animal to animal. Thus, a reliable measurement of melanophore responses must be based on a method which permits the calculation of an average response in a number of animals and statistical analysis of the data.

A photoelectric method of measuring melanophore responses *in vivo* has been previously reported by Thing (1952). He compared it with the microscopic method of estimating melanophore responses using Hogben's index (Hogben and Slome, 1928; Landgrebe and Waring, 1950). His conclusion that "both methods are equally suitable" is not supported by any statisical analysis. The melanophore indices of the microscopic method are based on an arbitrary scale and therefore cannot be treated statistically.

Thing's experimental procedure was different from that reported here in such aspects as: Adjusting doses on per weight basis; expressing responses as per cent change; and employing the two-hour-reflectance as a measure of response. Statistical analysis of our results shows that the employment of such procedures tends to increase the variability and thus reduce the precision of the method.

The present method takes into consideration all the known variables, and appropriate corrections are made, utilizing information obtained from the internal evidence of the results (such as adjusting for significant covariances).

Regarding the choice of response, the obvious practical advantages of using the one-hour-response would make it preferable to the peak-response for purposes of bioassay. Since the precision indices of the peak and one-hour-response regressions are not significantly different, the latter may be used without any loss of precision.

The variability of responses among animals as reflected in the variance of error is large considering the magnitude of the slope of the dose-response regression, thus resulting in a relatively high value for the degree of precision when compared to other bioassays (Bliss and Cattell, 1943). This in turn indicates that in assays relatively large number of animals per dose group would be required for a fair degree of accuracy.

The main advantage of the method presented here is that it provides objective, quantitative measurements which can be treated statistically, so that their variability may be analyzed and their precision estimated. This gives the results a certain known degree of accuracy which is not found in any of the *in vivo* methods now available for the measurement of melanophore responses. Furthermore, repeated tests, utilizing over 100

frogs, indicate that the dose-response regression line is relatively constant for several months.

SUMMARY

An objective photoelectric method for the quantitative measurement of melanophore expansion in frogs is described. The data are treated statistically to determine the precision and accuracy of the method.

Using an ACTH preparation as a source of melanophore expanding principle, a linear log dose-response relationship was obtained over the range of 0.064 to 6.4 μg. per frog. The slope, standard deviation, and degree of precision of the regression were relatively constant over a six month period.

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LIVER GLUCOSE-6-PHOSPHATASE ACTIVITY AND INTRACELLULAR DISTRIBUTION AFTER CORTISONE ADMINISTRATION[1]

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INTRODUCTION

THE behavior of liver glucose-6-phosphatase (G-6-Pase) in various physiological and pathological conditions has recently been under investigation in this laboratory. It was shown that in adult rats and mice, liver G-6-Pase activity increased in fasting (Weber and Cantero, 1954). On the other hand, G-6-Pase activity progressively decreased in the precancerous liver of 4-dimethylaminoazobenzene fed rats and it was almost completely absent in the developed hepatoma. The transplantable Novikoff rat hepatoma and various transplantable mouse tumors showed no G-6-Pase activity (Weber and Cantero, 1955). Studies in regenerating liver showed that this enzyme was not affected in the rapidly growing adult liver. On the other hand, almost no enzymatic activity was present in the 16 and 19 day old rat embryos (Weber and Cantero, 1955). These changes in the liver G-6-Pase activity under different physiological and pathological conditions have focussed our interest upon the factors which may influence the G-6-Pase reaction *in vivo*.

It has been shown that high doses of cortisone resulted in permanent diabetes in rat, characterized by hyperglycemia and glycosuria (Ingle, 1941). Insulin-resistant "cortisone diabetes" showing very high blood sugar levels and glycosuria has also been reported in human (Geller *et al.*, 1951). The mechanism of the action of cortisone in increasing blood sugar level has been attributed to increased gluconeogenesis. There is also evidence that certain phases of carbohydrate utilization (Colowick *et al.*, 1947) and glycogen breakdown (Kerppola, 1952) are inhibited. On the other hand, it was demonstrated that cortisone administration caused a striking increase in the rate of glucose production (Welt *et al.*, 1952). Since glucose production from the liver is related to the rate of hydrolysis of the glucose-6-phosphate (G-6-P) ester, it seemed of interest to study the enzyme G-6-Pase in the liver of animals treated with cortisone.

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MATERIALS AND METHODS

Animals and experimental groups. Young male Wistar rats (100 ± 10 gm. of weight) were used. The following experimental groups were set up.

(A) 1. Normal untreated fed rats.
2. Normal untreated 24-hour fasted rats.
(B) 3. Vehicle injected rats, fed until sacrifice.
4. Cortisone injected rats, fed until sacrifice.
(C) 5. Vehicle injected rats, fasted 24 hours before sacrifice.
6. Cortisone injected rats, fasted 24 hours before sacrifice.

There were four animals in each group and the whole series was repeated four times The animals were maintained on Purina Fox Chow and water *ad libitum*. The treated rats received daily intramuscular injections of 25 mg. of cortisone acetate (Cortone, Merck) for five days and were sacrified on the sixth day. The control animals were injected the same way with the vehicle, which contained only the suspending medium of cortisone, but no cortisone. The fasted animals were placed in individual cages with water available *ad libitum*.

Biochemical determinations. The animals were killed by a blow on the head and were decapitated and bled. The liver was exposed and a small sample for glycogen assay was immediately taken from the right lobe and placed into weighed test tubes containing 30 % KOH. Samples for histological studies were taken at the same time. The slides were stained with haematoxylin and eosin, PAS, and Sharlach.

The livers were quickly excised, pooled in a beaker standing on ice, chilled for five minutes, then blotted on filter paper and weighed. The spleens were also removed, blotted on filter paper and quickly weighed on a Roller-Smith Precision Balance. The pooled livers were passed through a plastic squeezer and from the obtained pulp 10 per cent homogenates were prepared in 0.25 M ice-cold sucrose (Allard *et al.*, 1954). The subsequent centrifugal separation of the cellular components: namely, nuclear, mitochondrial, microsomal and supernatant fractions followed the separation scheme of Schneider and Hogeboom (1950). The G-6-Pase activity was assayed according to Cori and Cori (1952) with slight modification (Weber and Cantero, 1955). The phosphorus was determined by the method of Fiske and Subbarow. The glycogen was determined by the method of Good, Kramer and Somogyi (1933) employing the Nelson's adaptation of the Somogyi method for glucose (1944). The enzymatic activities are expressed per wet weight, per nitrogen, per average cell basis, and also per liver weight/body weight ratio. The glycogen content is expressed as gm. % of wet weight liver and also as micrograms per average cell. The micro-Kjeldahl procedure was employed for total nitrogen determinations. The technique of cell counts is outlined in another publication (Allard *et al.*, 1955). All data represent the mean of four pools of livers each of four animals.

RESULTS AND DISCUSSION

Effect of cortisone on growth, spleen weight, liver weight and liver/body weight ratios. The effect of cortisone on growth of young animals is shown in Figure 1. The cortisone treated animals lose weight steadily from the second injection. By the sixth day the cortisone treated fed animals have lost 14% of their body weight, while the control animals have gained 23% during the same time period. The weight loss during the 24 hour fasting is not influenced by cortisone. The spleen weight of the cortisone treated animals decreased to about 25% of the control group. The marked decrease

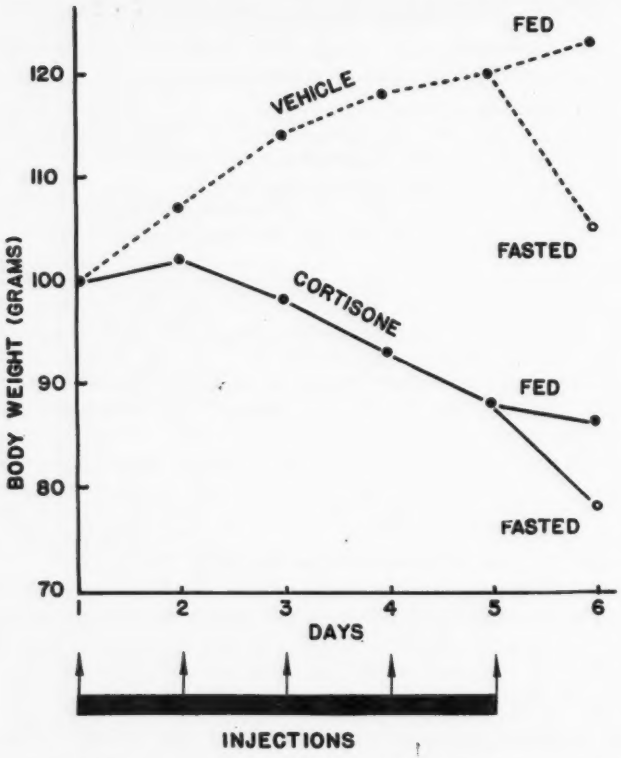


FIG. 1. The effect of cortisone on body weight. For five days the animals received daily injections of cortisone or vehicle respectively and food and water were available *ad libitum*. Fed: food and water available until sacrificed on sixth day. Fasted: food withdrawn on the fifth day and animals fasted 24 hours before sacrificed on the sixth day.

in spleen weight following cortisone administration confirms the data of Molonut *et al.* (1950). The effect of cortisone on liver weight and liver/body weight ratios is shown in Table 1. The 24-hour fasting causes a 40% decrease in the liver weight of normal rats. Vehicle injection does not influence this change in liver weight in fasting. Cortisone administration does not influence the liver weight of fed animals, but it inhibits the decrease in weight after fasting. The liver/body weight ratio decreases by 24% in normal animals after 24 hours of fast and this is not influenced by vehicle administration. Cortisone administration increases the liver/body weight ratios of fed rats by 50% above the vehicle injected ones.

Our finding of loss of body weight after administration of cortisone agrees with the findings of Kochakian *et al.* (1951). It is of interest to note that cortisone inhibited the decrease in liver weight and liver/body weight ratios in fasting animals since a similar phenomenon was described following x-irradiation by Ross and Ely (1951). The increase of liver/body

TABLE 1. LIVER WEIGHT AND LIVER/BODY WEIGHT RATIOS IN FED AND FASTED NORMAL, VEHICLE AND CORTISONE INJECTED RATS*

Experimental groups	Liver weight (gm.)		Liver/body weight ratio	
	Fed†	Fasted‡	Fed	Fasted
Normal	6.2 ± .2	3.8 ± .3	.049 ± .003	.037 ± .002
Vehicle§	6.1 ± .7	4.6 ± .4	.048 ± .003	.043 ± .006
Cortisone§	6.8 ± .3	6.0 ± .3	.078 ± .008	.069 ± .005
Per cent change after cortisone‖	111	130	163	160

* The mean values and standard deviations are given. The means represent the values of four pools each of four livers. The standard deviation = $\sqrt{\Sigma x^2/(n-1)}$. x = deviation from the mean, n = number of pools.

† Food and tap water *ad libitum* until sacrificed.

‡ Fasted for 24 hours before sacrificed. Tap water *ad libitum*.

§ Daily injections for five days, sacrificed on sixth day.

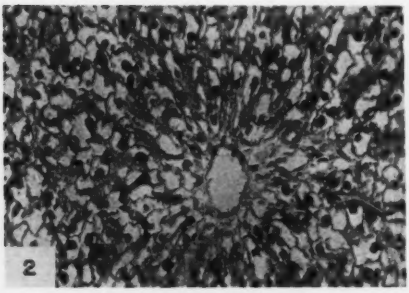
‖ The effect of cortisone is expressed in per cent changes; the values of the vehicle treated group are taken as 100 per cent.

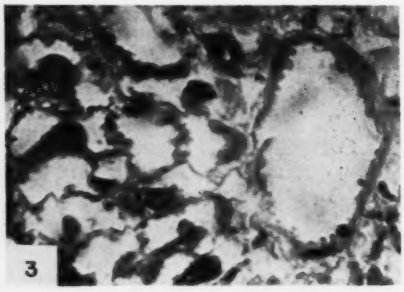
weight ratios in fed animals is due to the fact that while body weight decreased in the cortisone treated animals, the liver weight was maintained on the same level as in the vehicle injected ones. This relative enlargement of the liver is due to accumulation of glycogen and fat in the liver tissue (Roberts *et al.*, 1952; Einhorn *et al.*, 1954).

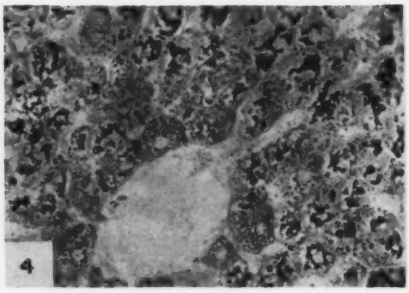
The histological picture of the cortisone treated rats shows the presence of apparent empty spaces (Figs. 2 and 3). Special stains, however, demonstrate that these spaces are filled with fat and glycogen. The striking accumulation of fat and glycogen in the liver of cortisone treated 24-hour fasted animals is shown in Figures 4–7. The histological pictures of normal 24-hour fasted rats are shown in Figures 8–11 for comparison.

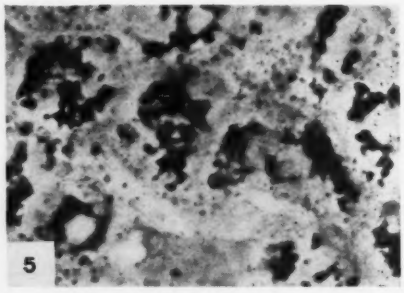
The effect of cortisone administration on liver glycogen of fed and fasted rats. Table 2 compares the liver glycogen values of vehicle and cortisone injected rats. The glycogen data are expressed as gm.% wet weight, as mg. of total liver glycogen and in micrograms of glycogen per average cell. Cortisone administration increased the glycogen content of fed livers to 217% when expressed as gm.% wet weight, to 248% as total liver glycogen, and to 317% in the average cell. The liver glycogen is depleted after 24 hours of fasting in the vehicle injected group. The total liver glycogen decreased in 24-hour fasting to 1–2% of the values of fed animals. The same decrease is observed when glycogen data are expressed on per cell basis. Cortisone administration in fasted rats resulted in values fifty to seventy times higher than in the vehicle injected fasted animals. The total liver glycogen in the cortisone treated fasting animals decreased only to 50% of the values of the cortisone injected fed animals. The glycogen content in the cortisone treated rats after a 24-hour fast was still higher than the glycogen content of the vehicle injected fed rats.

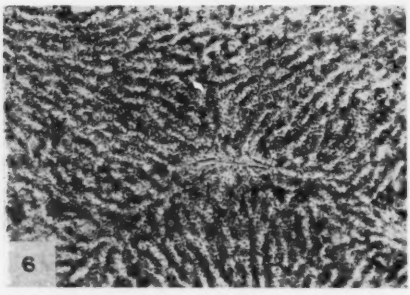
The glycogen data in fed and fasted cortisone injected animals confirm











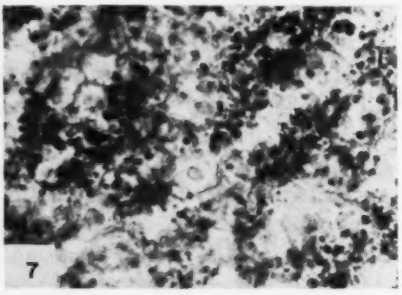


FIG. 2. Liver of cortisone injected rat. The animal was fasted 24 hours before sacrificed. The cytoplasm contains numerous apparently empty spaces H. and E. × 235.

FIG. 3. The same liver of fig. 2. H. and E. × 705.

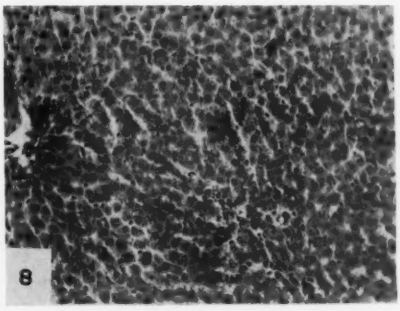
FIG. 4. The same liver of fig. 2. Picture shows that the "apparently empty spaces" in the cytoplasm are filled with glycogen. PAS. × 375.

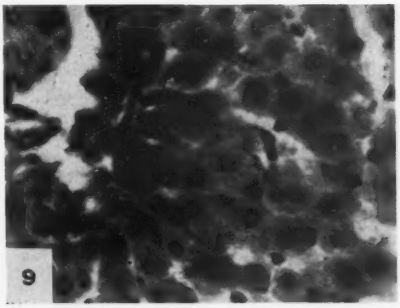
FIG. 5. The same liver of fig. 4. Glycogen deposition is mainly about the nucleus. PAS. × 705.

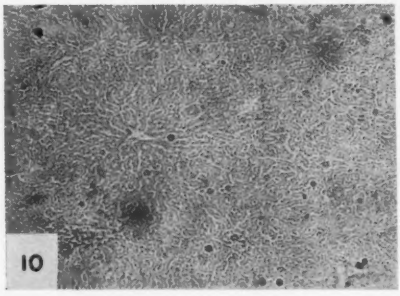
FIG. 6. The same liver of fig. 2. Shows very extensive fat deposition. Scharlach X 90.

FIG. 7. The same liver of fig. 6. Shows distinct fat droplets. Scharlach × 415.

the glycogenic effect of cortisone in the fasting rat reported by many authors as gram per cent values. When glycogen values are expressed on per cell basis the glycogenic effect of cortisone is even more striking. The glycogenic effect of cortisone administration in fasting animals is similar to the glycogenic effect of x-irradiation in fasted rats (Ross and Ely, 1951).







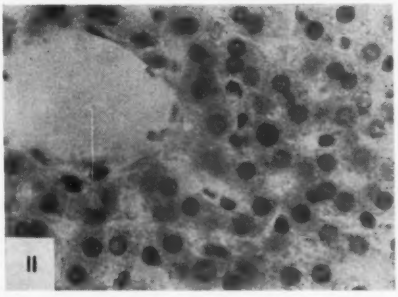


FIG. 8. Liver of 24 hour fasted normal rat. H. and E. × 200.

FIG. 9. The same liver of fig. 8. H. and E. × 375.

FIG. 10. The same liver of fig. 8. Minimal amounts of glycogen. PAS. × 80.

FIG. 11. The same liver of fig. 8. Almost complete absence of fat deposition. Scharlach and H. × 375.

This is in line with studies which demonstrated the mobilization of cortical hormonal reserves after irradiation (Hochman and Bloch-Frankenthal, 1953).

G-6-Pase activity and distribution in the liver of 24-hour fasted young rats. Table 3 compares the liver G-6-Pase activities of fed and 24-hour fasted rats. The G-6-Pase activities per wet weight and nitrogen are significantly increased in the homogenates, mitochondrial and microsomal fractions of fasted livers. The significant increase in liver G-6-Pase activity per wet weight and nitrogen in young rats after 24-hour fasting confirms previous

TABLE 2. THE EFFECT OF CORTISONE ADMINISTRATION ON LIVER GLYCOGEN CONTENT IN FED AND FASTED RATS*

Experimental groups	Gram per cent		Total liver glycogen (mg)		Per cell $\mu g \times 10^{-6}$	
	Fed	Fasted	Fed	Fasted	Fed	Fasted
Vehicle	5.9 ± 2.5	0.14 ± 0.04	371 ± 190	6.4 ± 2.5	211 ± 109	4.7 ± 3.2
Cortisone	13.4 ± 6.8	7.6 ± 4.4	921 ± 241	448 ± 167	669 ± 299	342 ± 161

* See Table 1 for statistical treatment of the data and definition of procedures.

TABLE 3. LIVER GLUCOSE-6-PHOSPHATASE ACTIVITY AND INTRACELLULAR DISTRIBUTION IN FED AND FASTED NORMAL RATS*

G-6-Pase activity	Homogenate		Nuclear		Mitochondrial		Microsomal		Recovery	
	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted
Per wet weight[1]	285 ± 33	443 ± 39 (155)†	52 ± 15	75 ± 8 (144)	67 ± 18	120 ± 7 (179)	130 ± 19	217 ± 32 (167)	86 ± 13	93 ± 2
Specific activity[2]	87 ± 12	125 ± 22 (144)	73 ± 15	89 ± 23 (122)	89 ± 14	141 ± 8 (158)	233 ± 62	338 ± 36 (145)		
Per cell[3] × 10^{-6}	10.9 ± 1.4	11.8 ± 1.4 (108)	1.9 ± 0.5	1.9 ± 0.1 (100)	2.5 ± 1.0	3.0 ± 0.5 (120)	4.8 ± 1.4	5.4 ± 1.2 (125)		

* See Table 1 for statistical treatment of the data and for procedures.
† The values in parentheses show the effect of fasting expressed as per cent change; the values of fed animals are taken as 100 per cent.
[1] Activity expressed as micrograms of phosphorus liberated in 15 minutes at 31° C. per 100 mg. liver wet weight at a pH 6.8.
[2] Activity expressed per mg nitrogen.
[3] Activity per average cell (Activity per unit wet weight/number of nuclei per same unit wet weight).

TABLE 4. THE EFFECT OF CORTISONE ADMINISTRATION ON LIVER GLUCOSE-6-PHOSPHATASE ACTIVITY IN THE HOMOGENATE AND CELLULAR FRACTIONS*

Glucose-6-phosphatase activity	Experimental groups	Homogenate		Nuclear		Mitochondrial		Microsomal		Recovery	
		Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted
Per wet weight[1]	Vehicle	271 ± 35	442 ± 76	30 ± 19	72 ± 40	70 ± 26	79 ± 25	140 ± 12	183 ± 52	92 ± 11	75 ± 15
	Cortisone	403 ± 46	366 ± 56	88 ± 14	81 ± 13	138 ± 26	136 ± 20	182 ± 16	142 ± 38	101 ± 5	98 ± 11
Specific activity[2]	Vehicle	90 ± 16	121 ± 22	47 ± 15	88 ± 20	102 ± 41	106 ± 30	226 ± 33	201 ± 11		
	Cortisone	146 ± 16	123 ± 17	110 ± 22	94 ± 19	249 ± 54	227 ± 40	418 ± 20	288 ± 68		
Per average cell[3] × 10^{-6}	Vehicle	9.9 ± 0.7	15.3 ± 2.2	1.2 ± 0.4	2.4 ± 0.4	2.6 ± 0.3	2.7 ± 0.5	5.2 ± 0.3	6.2 ± 1.0		
	Cortisone	19.3 ± 2.2	15.7 ± 1.6	4.2 ± 0.6	3.5 ± 0.1	6.6 ± 1.2	5.9 ± 0.9	8.8 ± 0.4	6.0 ± 1.0		

* See Table 1 for statistical treatment of the data and for procedures.
[1,2,3] For definition of the activity see Table 3.

findings of Weber and Cantero (1954), obtained in adult rats after 48-hour fast. The increased homogenate values correspond to increased activities demonstrated in all fractions on wet weight and nitrogen basis. When activities are expressed on per cell basis, however, no increase is found in the homogenates or fractions of 24-hour fasted young rats. This is taken as an indication that the G-6-Pase increase per wet weight and nitrogen was a reflection of the greater number of cells per gram.

G-6-Pase was found to be mainly concentrated in the microsomal fraction (50%). The activity was about equally (25%–25%) distributed in the nuclear and mitochondrial fraction. No G-6-Pase activity could be demonstrated in the supernatant. The recovery was usually between 80 and 100%. It was found that fasting did not cause any significant alteration in the percentage distribution of G-6-Pase in the liver cell (Table 5).

The distribution of G-6-Pase activity generally agrees with the findings of De Duve *et al.* (1954) since most of the activity is concentrated in the microsomal fraction. However, we found higher concentration of the activity in the mitochondrial fraction than De Duve and no G-6-Pase activity could be demonstrated in the supernatant fluid, whereas De Duve found small activities there. This discrepancy is probably a reflection of the difference in techniques of fractionation used. There is also a slight difference between the methods of assaying G-6-Pase. (See Addendum.)

The effect of cortisone on liver G-6-Pase activity and distribution. Table 4 compares the G-6-Pase activities in vehicle and cortisone injected rats. Cortisone caused a significant increase in the G-6-Pase activity in the homogenate of fed rats as compared to the vehicle. This increase is 49% on the basis of wet weight, 62% on the basis of nitrogen and 95% when expressed on per cell basis. The increased G-6-Pase activity of the homogenate of cortisone injected rats is reflected in the increase of the enzyme activity in all three particulate fractions and it is mainly due to increases in the activities in the nuclear and mitochondrial fractions. This increase could be due to an increased contamination from the microsomal fraction, but phosphatase and ribonuclease determinations in the nuclear fraction give no evidence of such an increased contamination (Allard *et al.*, 1955). However, the possibility of the contamination of the nuclear and mitochondrial fractions by microsomes cannot be completely excluded. It is felt that the increased activity in the nuclear and mitochondrial fraction is due to an increased enzymatic activity in the nuclei and mitochondria. The mitochrondrial activity increase is more marked than it seems from the data because the number of mitochondria decreased to half as a result of cortisone administration (Allard *et al.*, 1955).

Effect of cortisone in animals 24-hour fasted before sacrifice. There is no significant difference between the G-6-Pase activities of cortisone and vehicle treated rats in the homogenate, nuclear and microsomal fractions (Table 4). On the other hand, cortisone significantly increased the G-6-Pase

activity in the mitochondrial fraction. This increase is 72% on the basis of wet weight, 114% per nitrogen and 119% per cell basis. It is interesting to note that in animals which were fasted before sacrificed the vehicle treatment increased the G-6-Pase activity and cortisone did not increase this any further. It seems that the handling and injecting of the animals with the vehicle is not an entirely neutral influence and may act as nonspecific stress. The increased mitochondrial and nuclear activities in the cortisone treated animals are not reflected in the original homogenate.

The effect of cortisone on the per cent distribution of G-6-Pase in fed and fasted liver cell. The administration of vehicle did not cause any significant alteration in the percentage distribution of G-6-Pase as compared to normal uninjected fed and fasted animals. Cortisone caused an increase in the mitochondrial activity in both fed and fasted animals of 89% and 49% respectively. (See Table 5.)

In vitro studies. The effect of cortisone on liver homogenate was also investigated. It was found that the G-6-Pase activity of cell free homogenates was not influenced by the addition of cortisone even in high concentration. Preincubation (15 to 60 minutes) of liver homogenate with cortisone at 37°C also failed to affect G-6-Pase activity.

Liver G-6-Pase activity per 100 gm. body weight and blood sugar level. The enzymatic data presented so far have expressed the enzymatic activities in the homogenate and cell fractions on the basis of unit weight or per single cell. Since in this experiment important changes were recorded in the weight of the liver and of the body in the different experimental groups, it is necessary to express our data on the basis of liver/body weight ratio. This way of presenting the data also gives information concerning the total liver enzymatic activity for the whole body. Table 6 presents a comparison of the G-6-Pase activities expressed as total liver activity per 100 gm. body weight and the blood sugar levels in the different experimental groups.

TABLE 5. THE EFFECT OF CORTISONE ADMINISTRATION ON THE PERCENTAGE DISTRIBUTION OF THE GLUCOSE-6-PHOSPHATASE ACTIVITY IN THE LIVER CELL FRACTIONS*

Experimental groups	Percentage distribution of activity†							
	Nuclear		Mitochondrial		Microsome		Recovery	
	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted
Normal	13 ± 4	17 ± 1	24 ± 8	27 ± 1	46 ± 10	49 ± 3	88 ± 13	93 ± 2
Vehicle	15 ± 7	13 ± 5	18 ± 3	27 ± 11	42 ± 9	52 ± 6	75 ± 15	92 ± 11
Cortisone	22 ± 2	22 ± 2	34 ± 3	38 ± 6	46 ± 8	39 ± 7	102 ± 5	98 ± 11
Per cent‡	147	169	189	141	110	75		

* See Table 1 for statistical treatment of the data and for definition of procedures.
† There was no activity in the supernatant fluid.
‡ The effect of cortisone administration is expressed as per cent change; the values of the vehicle treated animals are taken as 100 per cent.

TABLE 6. COMPARISON OF BLOOD SUGAR LEVELS AND LIVER GLUCOSE-6-PHOSPHATASE ACTIVITY PER 100 GRAM BODY WEIGHT*

Experimental groups	Blood sugar (mg. per cent)		G-6-Pase activity per 100 g body weight	
	Fed	Fasted	Fed	Fasted
Normal	117 ± 18	77 ± 9	1450 ± 210	1670 ± 90
Vehicle	147 ± 17	93 ± 7	1280 ± 130	1880 ± 420
Cortisone	262 ± 87	144 ± 18	3150 ± 560	2530 ± 520

* See Table 1 for statistical treatment of the data and for definition of procedures.

The blood sugar level in fed animals depends on the intestinal absorption, peripheral utilization and the production of glucose from the liver by the G-6-Pase reaction. In fasting animals the blood sugar level depends on gluconeogenesis, peripheral utilization and production of glucose from the liver by the G-6-Pase reaction. These considerations indicate the importance of G-6-Pase reaction in the regulation of blood glucose level.

Table 6 shows the high blood sugar levels found after cortisone treatment. On the other hand, cortisone administration greatly increased the G-6-Pase activity above the level of vehicle injected animals. The highly increased liver G-6-Pase activity may be responsible, partly at least, for the high blood glucose levels in cortisone treated animals. This finding also may explain the report of Welt *et al.* (1952) on the great increase in the rate of glucose production after cortisone treatment. The finding of high liver G-6-Pase levels in hyperglycemic animals also agrees well with the recent report of Ashmore *et al.* (1954) on increased liver G-6-Pase activity in alloxan diabetic rats.

The demonstration of the effect of cortisone on liver G-6-Pase activity brings up the possibility that the adrenal cortex may play a role in the physiological maintenance and regulation of this enzyme. The study of the effect of adrenalectomy and hypophysectomy on liver G-6-Pase activity is in progress.

SUMMARY

Cortisone inhibited body growth, decreased spleen weight and increased liver weight/body weight ratio.

The histological studies of the livers of cortisone treated rats demonstrated extensive cytoplasmic vacuolization and heavy deposition of glycogen and fat

Cortisone caused a marked increase in the G-6-Pase activity of the homogenate, nuclear, mitochondrial and microsomal fractions per unit weight, nitrogen and average cell. The total liver G-6-Pase activity per 100 gram body weight was also highly increased. High blood sugar levels were present in these cortisone treated animals.

The percentage intracellular distribution of G-6-Pase in the liver homogenate was not affected by fasting or cortisone administration.

The possible role of liver G-6-Pase in the production of hyperglycemia after cortisone administration was discussed.

ADDENDUM

Since this manuscript was prepared for publication Langdon and Weakley have published (*J. Biol. Chem.* **214:** 167. 1955) a report on the effect of fasting and hormones on liver glucose-6-phosphatase. The dose of cortisone used by these authors was 10 times smaller than the one used in the present paper and correspondingly they demonstrated a less impressive increase in liver glucose-6-phosphatase. It is of interest to note that these authors found the same intracellular distribution of glucose-6-phosphatase activity as described in the present paper.

Acknowledgments

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THE METABOLISM OF I^{131} BY SURVIVING SLICES OF RAT MAMMARY TISSUE[1]

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TWO juxtaposed, dissociable processes participate in the thyroidal manufacture of hormone. First, the thyroid transports inorganic iodide against a concentration gradient (VanderLaan and VanderLaan, 1947; Taurog, Chaikoff and Feller, 1947). Secondly, the accumulated anion is employed in organic iodinations within the thyroid gland (Morton and Chaikoff, 1943; Schachner, Franklin, and Chaikoff, 1944). Isolated similar functions have been reported in extrathyroidal sites. For example, concentration differentials for ionic iodide are regularly established in saliva and in gastric secretions (Lipschitz 1929; Schiff, Stevens, Molle, Steinberg, Kumpe and Stewart, 1947). As yet, an extrathyroidal model displaying both properties has not been described.

In 1944, the classical experiments of Keston demonstrated the presence of soluble iodinating enzymes in unpasteurized milk. Van Middlesworth, Tuttle, and Haney (1954) have shown that such iodinations of milk proteins may also occur in vivo. Recently, Honour, Myant, and Rowlands (1952) have reported that inorganic iodide is concentrated by the lactating human breast. These combined data would suggest that in mammary tissue, as in the thyroid, an association of mechanisms may co-exist for concentrating iodide and for organic iodinations. Accordingly, these properties have been examined in surviving mammary slices. Preliminary results are outlined in the present communication.

METHODS

Tissues were obtained from female Sprague Dawley rats. Only one animal was employed in each experiment. Lactating rats were permitted to nurse their young until immediately prior to sacrifice. Animals were killed by a sharp blow on the head. Right and left inferior mammary glands were rapidly removed, freed of adherent fascia, and

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[2] Fellow of the American Cancer Society, Inc. recommended by the Committee on Growth of the National Research Council, 1953–1955.

placed in chilled saline. Mammary slices, 0.3–0.4 mm. in thickness, were prepared with a Stadie-Riggs microtome and washed in three successive portions of fresh saline to remove preformed milk. Other tissues were treated similarly. Slices were briefly blotted, weighed on a torsion balance, and introduced in 150 ± 15 mg. amounts into Warburg vessels. In studies with thyroid tissue, the individual lobes were not sectioned further, but were simply weighed and incubated directly.

Warburg techniques and methods of radioactive assay have been described previously (Freinkel and Ingbar, 1955a). Krebs-Ringer-Phosphate (KRP) (Umbreit, Burris and Stauffer, 1949) buffered at pH 7.4 and containing 150 mg. % glucose was employed as suspending medium. 2.7 cc. of non-radioactive KRP were placed into the main compartment of Warburg vessels; 0.3 cc. of the same KRP supplemented with 10 μc./ml. of carrier free inorganic I^{131} were placed into the side-arm. To inhibit inorganic iodinations, 1×10^{-3} M concentrations of 1-methyl-2-mercaptoimidazole[3] were incorporated into the KRP (KRP-Tap. 10^{-3} M) in some studies.

Tissues were incubated at 38° C. in an atmosphere of 100% oxygen. Oxygen consumption was measured by direct manometry (Umbreit, Burris and Stauffer, 1949) at ten minute intervals. A Q_{O_2} was derived for each flask as cu. mm. O_2 per hour per mg. of initial wet weight. Thyroidal Q_{O_2} could not be assessed accurately since the limited amount of tissue (10 to 20 mg. per flask) precluded adequate manometric excursions.

Exactly two hours following the introduction of radioactivity from the side-arms, tissues were removed from the flasks, blotted, and weighed on a torsion balance. Suspending solutions were centrifuged immediately to remove cellular detritus. Radioactivity within the tissues was partitioned into two fractions on the basis of differential solubility in trichloracetic acid (TCA): Slices were homogenized—extracted in ground glass homogenizers containing 2.0 ml. of 10% TCA. Homogenates were quantitatively transferred and centrifuged. The precipitates were resuspended, and successively washed, and centrifuged with 5.0 ml. portions of 2.5 %TCA and distilled water. All supernates were alkalinized and pooled into a single "TCA-soluble" fraction. The precipitates or "TCA-insoluble" fractions were digested in 2.0 N NaOH. In two experiments, the residual radioactivity within the suspending medium was also fractionated into "TCA-soluble" and "TCA-insoluble" moieties.

All tissue radioactivity was related to final wet weight. The KRP studies were designed to provide some index of organic iodinations and herein, the percentile incorporation of I^{131} into "TCA-insoluble" moieties was expressed as:

$$\frac{\text{TCA-Insoluble counts/100 mg. of Tissue}}{\text{Total Reovered Counts (tissue + medium)}} \times 100$$

The transport of inorganic iodide was investigated in the KRP-Tap. 10^{-3} studies and, herein, concentration gradients for ionic I^{131} (slice/medium ratios or S/M I^{131}) were expressed as:

$$\frac{\text{TCA-Soluble counts/gm. of Tissue}}{\text{Counts/ml. of Medium}}$$

RESULTS

During incubations for two hours in KRP containing 150 mg. % glucose, both lactating and non-lactating mammary slices incorporated sig-

[3] 1-methyl-2-mercaptomidazole: "Tapazole"—generously supplied by Dr. C. D. Hines of the Lilly Research Laboratories.

TABLE 1. THE INFLUENCE OF ANTITHYROID DRUGS ON RESPIRATION AND ORGANIC IODINATIONS BY SURVIVING RAT TISSUES

Exp. No.	Status of animal	Tissue	TCA-insoluble radioactivity (% recovered I^{131}/100 mg.)		Q_{O_2}	
			KRP	KRP-Tap. 10^{-3}	KRP	KRP-Tap. 10^{-3}
1	Non-pregnant; non-lactating	Breast	1.18	0.08	0.30	0.27
		Kidney	0.14	0.13	2.12	2.04
		Diaphragm	0.14	0.10	0.77	0.87
		Thyroid	24.01	0.06	—	—
7	Pregnant; term	Breast	3.3	0.02	0.74	0.76
8	Pregnant; term	Breast	1.98	0.01	0.57	0.60
		Breast (boiled)[1]	0.02	0.03	0	0
		Thyroid	31.50	0.01	—	—
9	6 Days post-partum; Lactating	Breast	2.14	0.05	1.18	1.08
		Breast (boiled)	0.01	0.02	0	—
		Thyroid	51.35	0.06	—	—

[1] Slices boiled for ten minutes prior to incubation.

nificant amounts of I^{131} into moieties which were insoluble in trichloracetic acid (Table 1). Concurrently incubated thyroid glands from the same donor animals displayed a greater capacity for organic iodinations. In two experiments, release of significant amounts of TCA-insoluble I^{131} from the mammary slices into the suspending KRP could not be demonstrated. No attempt was made to characterize the TCA-insoluble radioactivity within the breast tissue. The formation of TCA-insoluble radioactivity could be inhibited by boiling mammary slices prior to incubation or by the inclusion of 1-methyl-2-mercaptoimidazole at a final concentration of 1×10^{-3} M in the suspending medium. Q_{O_2} of surviving mammary slices was not appreciably altered by 1-methyl-2-mercaptoimidazole.

In KRP-Tap. 10^{-3}, radioactivity was entirely soluble in TCA and was identified as inorganic I^{131} by butanol extraction and by chromatography with such solvent systems as butanol-acetic acid or collidine-ammonia (Taurog, Tong and Chaikoff, 1950). Slice/medium gradients for inorganic I^{131} were less than 1.0 following incubation of rat hemidiaphragms or slices of rat kidney cortex. In three experiments, mammary slices derived from non-pregnant, non-lactating rats or from pregnant rats at term also failed to establish S/M I^{131} ratios greater than 1.0. Contrariwise, gradients exceeding 1.0 were obtained in seven of eight experiments with mammary slices derived from nursing rats, 4 to 16 days postpartum (Table 2). Eight to fifteen-fold greater concentration differentials were established by the thyroid glands of the same animals. In the mammary slices, as in surviving thyroid tissue (Slingerland, 1955; Freinkel and Ingbar, 1955b), concentra-

TABLE 2. THE EFFECT OF ANIONS AND 2,4-DINITROPHENOL UPON IODIDE TRANSPORT BY LACTATING MAMMARY SLICES

Exp. No.	Inhibitor	Concentration (M)	Slices[1]	
			S/M I^{131}	Q_{O_2}
4	—	—	2.29	0.89
	DNP	2.5×10^{-5}	0.88	0.43
	DNP	1×10^{-5}	1.51	0.78
	ClO_4^-	1×10^{-2}	0.64	0.81
9	—	—	2.55	1.08
	Boiled[2]	—	0.94	0
	DNP	2.5×10^{-5}	1.46	0.93
	ClO_4^-	1×10^{-2}	0.81	1.12
11	—	—	1.75	0.57
	SCN^-)	1×10^{-2}	0.83	0.48
	DNP	2.5×10^{-5}	1.01	0.41

[1] Slices were suspended in KRP-Tap. 10^{-3} M. In experiments with anions, total osmolarity was maintained at 310 mOs/L by appropriate adjustment in the NaCl of the KR solution.

[2] Mammary slices were boiled for ten minutes prior to incubation.

tion differentials for inorganic I^{131} could be depressed by anions such as thiocyanate and perchlorate, or by metabolic inhibitors such as 2, 4 dinitrophenol (DNP) (Table 2).

DISCUSSION

It has been demonstrated that surviving mammary tissues can incorporate inorganic I^{131} into moieties which are insoluble in trichloracetic acid. Although fractionation with TCA may coprecipitate anionic I^{131}, repeated washings were instituted to minimize this possibility. Furthermore, the addition of 1-methyl-2-mercaptoimidazole or the boiling of tissue slices prior to incubation inhibited the formation of TCA-insoluble radioactivity. Thus it seems likely that the phenomenon represents, at least in part, true organic iodination.

It has also been shown that lactating mammary slices can transport inorganic iodide against a concentration gradient. The absolute magnitude of both organic iodinations and of iodide transport may have been underestimated in the present studies. For example, in the KRP system, the possible formation of organic iodinated compounds which are soluble in TCA (Taurog, Tong and Chaikoff, 1951) has not been investigated. Moreover, loss of TCA-insoluble radioactivity into the medium by leaching or secretion cannot be wholly excluded on the basis of two negative experiments. Similarly, the slice/medium differentials in KRP-Tap. 10^{-3} may constitute only minimal approximations of actual transport. Conceivably, with continued secretory activity *in vitro*, radioactivity within the slice at any instant in time may represent only the net balance between ionic influx and egress.

Despite these limitations, the coexistence of mammary mechanisms for the concentration and organification of ionic iodide is readily apparent. The interrelation of these activities within the breast is less clearly defined. Organic iodinations occur in non-lactating slices whereas slice/medium differentials exceeding 1.0 could be demonstrated only in tissue derived from lactating animals. In the absence of some quantitative technique for assessing functional glandular mass, correlations between iodide transport, iodinations, and secretory activity must be deferred. Thus, it cannot be said whether the apparently diminished gradients of nonlactating tissues are of physiological significance or merely the result of dilution of mammary mass by non-secretory components.

Mammary S/M^{131} ratios can be impaired by perchlorate and thiocyanate. As in the thyroid, this anionic inhibition is effected without significant depression of oxygen consumption (Slingerland, 1955; Freinkel and Ingbar, 1955b). Concentration gradients in lactating breast tissue can also be inhibited by concentrations of 2,4 dinitrophenol which dissociate oxidation from phosphorylation in other systems (Loomis and Lipmann, 1948; Cross, Taggart, Covo and Green, 1949). In mammary slices, the action of 2,4 dinitrophenol is accompanied by a slight reduction of Q_{O2}; however transport performance is depressed to a greater extent. The preliminary evidence would suggest that mammary transport of iodide simulates that of the thyroid gland, both in anionic interactions and in dependence upon energy derived from oxidative phosphorylations (Freinkel and Ingbar, 1955b). The implications of these similarities are beyond the scope of the present communication. Moreover, the available data do not indicate whether the juxtaposed "trapping" and "binding" mechanisms within the mammary gland bear more than superficial resemblance to their thyroidal counterparts. Nonetheless, the surviving mammary slice may well afford an excellent preparation for studying the comparative physiology of these functions in extrathyroidal sites.

SUMMARY

Surviving slices of rat mammary tissues have been employed to study extrathyroidal iodine metabolism in the Warburg apparatus.

Lactating and non-lactating slices incorporated inorganic I^{131} into moieties which were insoluble in trichloracetic acid. Organic binding could be inhibited by 1-methyl-2-mercaptoimidazole or by boiling the tissues prior to incubation.

In media containing sufficient 1-methyl-2-mercaptoimidazole to inhibit organic iodinations, lactating mammary slices established slice/medium differentials for inorganic I^{131} which exceeded 1.0. Concentration gradients could be depressed by monovalent anions such as thiocyanate or perchlorate, or by metabolic inhibitors such as 2,4 dinitrophenol.

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THE RESPONSE OF THE ADRENAL CORTEX OF THE RAT TO DIETARY SALT RESTRICTION AND REPLACEMENT[1]

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SEVERAL investigations have been concerned with the effects of altered sodium or potassium intakes on the adrenal cortex of the rat (Orent-Keiles, Robinson and McCollum, 1937; Badinez and Croxatto, 1948; Deane, Shaw and Greep, 1948; and Bergner and Deane, 1948). Most of these studies have evaluated the changes induced by deficient diets upon the widths and cytochemistry of the zona glomerulosa. When rats are kept on a diet restricted as to sodium and chloride, there is an increase in the size of the zona glomerulosa and a decrease in lipid droplets. The object of this investigation was to determine with what rapidity the zona glomerulosa returns to normal after a rat has been on a salt free diet and is allowed to ingest salt *ad libitum*. In addition to histological studies, chemical determinations for ascorbic acid and cholesterol in the adrenals of salt deficient rats were made.

MATERIALS AND METHODS

Ninety-six young male albino rats were used in the experiments, most of them being paired litter mates. Fifty-three were placed on the very low sodium diet* of Orent-Keiles, Robinson and McCollum (1937), and forty-three on the same diet added to which was their salt mixture No. 19 (Orent-Keiles, Robinson and McCollum, 1937). Of the fifty-three experimental animals, eight were later given *ad libitum* in a 1% solution and sacrificed at 24 and 48 hours after being on a salt deficient diet for twenty-eight days. The remainder of the experimental animals were sacrificed at intervals of 4, 7, 15, 27 and 43 days while on the low salt diet. When sacrificed, care was exercised to handle them so as to prevent as much as possible the "alarm reaction": the rats were anesthetized with Nembutal and decapitated. The adrenal glands were dissected free and weighed. The ascorbic acid content was determined by the method of Roe and Keuther (1943) and the total cholesterol content by the method Sperry and Webb (1950). The adrenals were frozen, sectioned and stained with sudan IV. Paraffin sections were stained with hematoxylin and eosin.

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* Diet contained 0.0047 gm. % sodium by analysis.

TABLE 1. THE EFFECT OF A SALT FREE DIET UPON THE ADRENAL WEIGHT OF THE RAT

	No. of days of exp.	No. of animals	Wt. one adrenal / 100 gm. rat wt.	Per cent change
control	4	5	6.84	
experimental	4	5	8.11	(+15.7)
control	7	2	11.7	
experimental	7	2	13.15	+11.0
control	15	2	8.6	
experimental	15	1	8.9	+ 0.34
control	27	7	5.86	
experimental	27	8	7.79	*+24.8*
control	43	1	8.0	
experimental	43	1	6.5	−18.75

Figures in italics indicate $P < 0.05$ (from table of t values); in parenthesis, $P = 0.1 - 0.05$.

TABLE 2. THE EFFECT OF A SALT POOR DIET AND REPLACEMENT OF SALT UPON TOTAL CHOLESTEROL CONTENT OF THE ADRENAL GLAND OF THE RAT

	No. of days of exp.	No. of animals	Mg./100 gm. gland	Per cent change
control	7	2	3.82	
experimental	7	1	2.40	−37.17
control	15	2	4.94	
experimental	15	2	3.68	−30.43
control	27	3	3.60	
experimental	27	4	2.53	*−29.72*
control	43	1	2.62	
experimental	43	1	2.29	−12.60
experimental	28	4	4.29	
salt ad lib.	28	4	5.33	*+24.2*

Figures in italics represent $P < 0.05$.

TABLE 3. THE EFFECT OF A SALT FREE DIET UPON THE ASCORBIC ACID CONTENT OF THE ADRENAL GLAND OF THE RAT

	No. of days of exp.	No. of animals	Mg./100 gm. gland	Per cent change
control	4	5	600	
experimental	4	5	563	− 6.17
control	7	2	528	
experimental	7	2	460	−12.88
control	15	2	527	
experimental	15	2	467	−11.39
control	27	6	565	
experimental	27	6	535	− 5.31
control	43	1	435	
experimental	43	1	601	+27.62

Differences were not significant.

RESULTS

There was no appreciable difference in the rate of weight gain between the control and experimental animals during the period of observation. The adrenal weights per 100 gm. of rat are shown in Table 1. This ratio was greater in the experimental as compared to the control animals. In Table 2, a decrease in the cholesterol content on the adrenal gland of rats on a low sodium diet is indicated; approximately a 25% reduction was observed. In one animal sacrificed at 43 days, the change was smaller. In Table 3, the ascorbic acid content of the adrenal is shown. Except for the one animal killed after 43 days, the amount appeared to be somewhat reduced, in keeping with the findings of Danford and Herrin (1951). The changes in weight and cholesterol were statistically significant ($P<0.05$); those in ascorbic acid, while showing a trend, were not.

In order to ascertain the effects of salt replacement, fourteen animals were studied: two on a normal diet were used as controls, twelve were placed on a low-sodium diet for 28 days, and eight of these received 1% salt in their drinking water for 24 to 48 hours prior to necropsy. Serum sodium, potassium, and chloride did not show alterations of significant degree. There was an increase in total cholesterol content of the adrenal

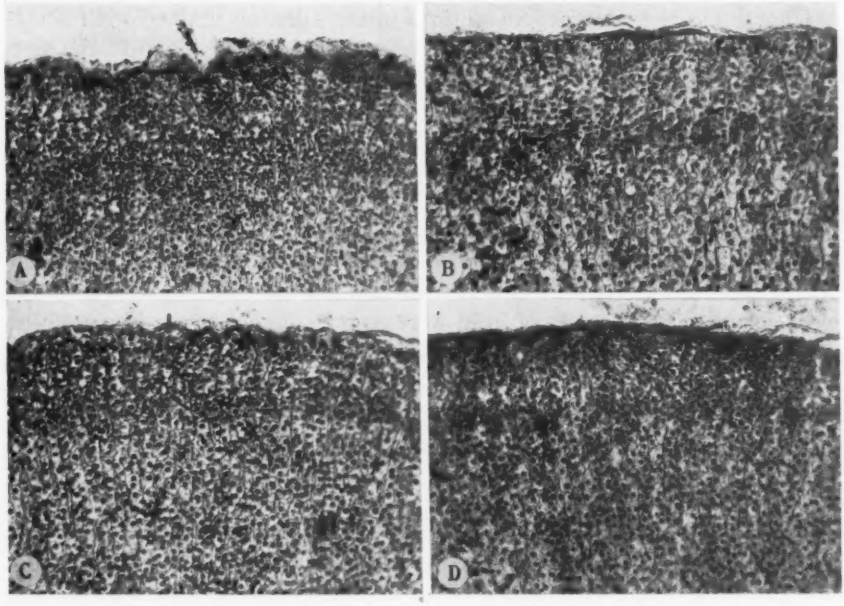

FIG. 1. The adrenal cortex of litter mates used in one group of experiments; paraffin sections stained with hematoxylin and eosin showing the width of the zona glomerulosa. The same magnification was used in all photomicrographs. A: Control. Sodium chloride *ad libitum*. B: sodium poor diet for 28 days. C: sodium poor diet for 28 days and sodium chloride *ad libitum* for 24 hours. D: sodium poor diet for 28 days and sodium chloride *ad libitum* for 48 hours. See text for explanations.

TABLE 4. EFFECT OF REPLETION OF DIETARY SALT ON ZONA GLOMERULOSA

	Rat No.	Hypertrophy (H & E stain)	Hypertrophy (Sudan stain)	Fat droplets (Sudan stain)	Total +
Controls	8	0−+	0	+	
	12	0−+	0	0	2
Diet alone 28 days	5	4+	4+	4+	
	6	4+	4+	4+	
	9	4+	3+	+	
	10	4+	3+	0	39
Salt 24 hrs.	7	3−4+	3+	0	
	11	3+	2+	+	
	13	3−4+	3+	4+	
	14	3−4+	4+	4+	34½
Salt 48 hrs.	1	2+	0	0	
	2	2+	2+	2+	
	3	2+	3+	3+	
	4	2+	2−3+	2−3+	23

Note: These impressions were obtained by examination of the microsections without knowledge of the regimen to which each rat had been subjected.

glands from an average of 4.29 mg./100 gm. of gland in four animals on the low-salt diet to 5.33 mg./100 gm. of gland in another four animals after 48 hours of salt replacement; values of all animals increased.

Changes in the microscopic sections of the adrenals stained with hematoxylin and eosin occurred in all animals when salt was added to the diet. Figure 1B shows the typical changes in the zona glomerulosa of a rat fed a diet low in sodium chloride as compared to a control rat fed the same diet with added salt (Fig. 1A). Figure 1C shows the adrenal cortex of a rat on salt *ad libitum* for 24 hours following a 28-day period of restricted salt. Figure 1D shows the appearance of the adrenal cortex of a similar rat 48 hours after the ingestion of salt. Within 24 hours there occurred a detectable diminution in the size of the zona glomerulosa and after 48 hours the zona glomerulosa had returned further toward normal. The spaces which were occupied by droplets of lipid were seen to change toward normal appearance within 48 hours when observed in sudan-stained sections. Without knowing the treatment to which these fourteen rats had been subjected, it was possible to pick out each section accurately from the microscopic appearance of the section and to place it in the appropriate group without error (Table 4).

DISCUSSION

Much has been written concerning the mechanism of hormone production and control in the zona glomerulosa of the rat. Recently a study of the adrenal cortices of hypertensive patients, by Peschel and Race (1954), showed that the zona glomerulosa of those on a rice diet was increased significantly in size in 24% of their cases. In addition, the fat content was decreased when the width of the zona glomerulosa was greater than normal.

From the present experiments one is impressed at the rapidity with

which the adrenal cortex may react to varying concentrations of electrolytes in the diet. After four days of salt deprivation, there are changes seen which are maintained for 43 days, the longest an experiment continued. When rats were allowed to take salt *ad libitum*, reversal toward normal was observed within 24 hours which was practically complete in 48 hours.

From these and others' observations (Deane, Shaw and Greep, 1948; and Danford and Herrin, 1951), it would appear that the increase in the width of the zona glomerulosa indicates increased activity and stimulation, perhaps because of a low plasma sodium concentration or a low plasma sodium/potassium ratio as shown by Deane, Shaw and Greep, (1948). In addition to the histochemical studies, ascorbic acid determinations and cholesterol contents of the adrenals indicate that there is a definite correlation between activity and these parameters, cholesterol content falling significantly and ascorbic acid less so.

SUMMARY AND CONCLUSION

The effect of a diet low in sodium chloride, with later replacement of salt, on the adrenal cortex of albino male rats was studied by histological and chemical methods, including content of ascorbic acid and cholesterol.

The histological findings were in keeping with studies previously described by other investigators and indicated an increase in the width of the zona glomerulosa and a decrease in lipid droplets.

The cholesterol content of the adrenal gland diminished with increased activity, while the ascorbic acid content diminished to a less significant degree.

Replacement of dietary salt caused within 24 hours a partial return to a normal appearance and at 48 hours the adrenal cortex appeared practically normal. The cholesterol content increased.

It is assumed that the low dietary sodium and chloride caused a stimulation of the zona glomerulosa which was rapidly reversible when salt was supplied.

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EFFECT OF PLASMA FROM HYPOPHYSEAL-PORTAL VESSEL BLOOD ON ADRENAL ASCORBIC ACID[1]

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THERE is lack of unanimity of opinion regarding the relative significance of different factors in the regulation of secretion of the adrenocorticotrophic hormone (ACTH) (Long, 1952). Much suggestive evidence, however, implicates the hypothalamus as being of importance, perhaps by way of a neurohumoral substance (Hinsey and Markee, 1933 and Green and Harris, 1947). The existence of vascular connections, a portal system, transporting blood from the hypothalamus to the anterior lobe of the pituitary is established (Wislocki and King, 1936; Green and Harris, 1947, 1949; and Xuereb *et al.*, 1954). Harris and Jacobsohn (1952) and Jacobsohn (1954) using pituitary transplants in the rat and rabbit respectively have suggested that function of the anterior lobe is dependent upon vascular connections between the pituitary and median eminence. Although results have varied somewhat, destructive lesions in different sites of the hypothalamus have been reported by several investigators to inhibit the release of ACTH by the experimental animal in response to stressful conditions (De Groot and Harris, 1950; Hume and Wittenstein, 1950; Porter, 1953; McCann, 1953; McCann and Sydnor, 1954; Ganong and Hume, 1954; and Laqueur *et al.*, 1955).

By use of a technic (Porter *et al.*, 1955) which permits one to obtain blood from the hypothalamico-hypophyseal portal vessels, it is possible to test for the presence of a neurohumoral agent in the blood draining the hypothalamus. This report deals with the effect of the plasma of this blood on adrenal ascorbic acid of intact and hypophysectomized rats.

MATERIALS AND METHODS

The plasma used in these experiments was obtained from blood of twelve mongrel dogs of both sexes varying in weight from 7 to 19 kg. Blood for experimental purposes was obtained from the hypothalamico-hypophyseal portal vessels (portal vessels); whereas, control blood was obtained from the common carotid artery. The procedure employed in obtaining blood from the portal vessels of these animals was as follows: All animals were anesthetized with chloralose-urethane (5% chloralose and 50% urethane in H_2O), using 50 mg. of chloralose and 500 mg. of urethane per kg. body weight.

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The anesthetic was administered intravenously over a period of 30 to 45 minutes, using intermittent injections. The pituitary was exposed by way of the temporal route. After all bleeding was stopped, the animal was heparinized (5 mg. of heparin per kg. body weight) and then the pituitary removed by aspiration. The blood which then accumulated in the sella from the broken portal vessels was removed by aspiration and temporarily retained in an ice bath. At the end of the collection period, which usually lasted 2½ to 4 hours, control blood was obtained from the common carotid artery. Plasma from each blood sample was removed following centrifugation and stored in the frozen state if not used immediately.

The effect of these plasma samples on adrenal ascorbic acid was measured using adult, male, albino rats of both the Sprague-Dawley and the Holtzman strains. The types of animal preparations employed in these experiments were (1) the hypophysectomized rat and (2) the hydrocortisone-inhibited, intact rat. The hypophysectomized rats were prepared by the parapharyngeal approach and were used in the adrenal ascorbic acid depletion test 22 to 24 hours after hypophysectomy (Sayers, Sayers, and Woodbury, 1948). Gross examination of the sella area was performed at autopsy and results from all animals showing incomplete operations were discarded. Intact rats which had received injections of hydrocortisone acetate in saline were also used in the adrenal ascorbic acid depletion test. Various adrenocortical steroids or extracts containing these substances are known to inhibit the release of ACTH by the anterior lobe (Ingle *et al.*, 1937, 1938; Sayers and Sayers, 1945, 1947; Gray and Munson, 1951; Abelson and Baron, 1952; and Sydnor *et al.*, 1955). Long (1955) has observed that hydrocortisone administered intraperitoneally 4 hours prior to unilateral adrenalectomy inhibits adrenal ascorbic acid depletion in the intact rat. Data presented in Table 1 confirm these observations. Hydrocortisone acetate administered intramuscularly in the quantities used was ineffective in adequately inhibiting adrenal ascorbic acid depletion following unilateral adrenalectomy. However, when hydrocortisone acetate was administered intraperitoneally, inhibition was complete. For the remaining experiments in which intact rats were used, 6 mg./100 gm. body weight of hydrocortisone acetate in saline was given intraperitoneally 4 hours prior to the removal of the first adrenal. Immediately following removal of the first adrenal, 2 ml. of the test plasma was given intravenously via the tail vein. In experiments where a total of 4 ml. per rat was used, a second injection of 2 ml. was given 30 minutes after the first. The second adrenal was removed ninety minutes following the first injection.

TABLE 1. EFFECT OF UNILATERAL ADRENALECTOMY ON ADRENAL ASCORBIC ACID IN THE RAT FOLLOWING INJECTION WITH HYDROCORTISONE ACETATE

No. of rats	Treatment (mg. hydrocortisone acetate per 100 gm. body wt.)	Time interval between injection and removal of left adrenal (first adrenal)	Ascorbic acid concentration, left adrenal, mg./100 gm. (mean)	Ascorbic acid concentration, right adrenal, mg./100 gm. (mean)	Difference in ascorbic acid concentration (Left adrenal minus right adrenal) (mg./100 gm.) Mean and S.E.
2	1 i.m.*	16–18 hrs.	408	193	−215 ± 42.0
3	2 i.m.	16–18 hrs.	331	182	−149 ± 28.0
3	1 i.m.	28–30 hrs.			
	4 i.m.	4– 6 hrs.	433	255	−178 ± 23.5
3	4 i.p.†	4 hrs.	312	306	− 6 ± 7.0
3	5 i.p.	4 hrs.	378	375	− 3 ± 15.0
3	6 i.p.	4 hrs.	360	363	+ 3 ± 7.3
6	7 i.p.	4 hrs.	337	351	+ 14 ± 8.5

* i.m. = intramuscularly.
† i.p. = intraperitoneally.

TABLE 2. EFFECT OF BLOOD PLASMA FROM HYPOTHALAMICO-HYPOPHYSEAL PORTAL VESSELS OF THE DOG ON ADRENAL ASCORBIC ACID OF HYDROCORTISONE-INHIBITED, INTACT RATS

Number of rats	Weight of rats (gm.)	Initial treatment (Hydrocortisone acetate, i.p.)	Ascorbic acid concentration, left adrenal (at 0 time) (mg./100 gm.) (mean)	Final treatment (blood plasma, i.v.)	Ascorbic acid concentration, right adrenal (at 90 min.) (mg./100 gm.) (mean)	Difference in ascorbic acid concentration (left adrenal minus right adrenal) (mg./100 gm.)
8	243 ± 10*	6 mg./100 gm. body weight	364	*0 min.*—2 ml. plasma from carotid artery	373	+ 9 ± 5.4
7	252 ± 14	6 mg./100 gm. body weight	391	*0 min.*—2 ml. plasma from portal vessels	385	− 6 ± 17.8
15	234 ± 8	6 mg./100 gm. body weight	353	*0 min.*—2 ml. plasma from carotid artery *30 min.*—2 ml. plasma from carotid artery	359	+ 6 ± 4.1
17	261 ± 8	6 mg./100 gm. body weight	351	*0 min.*—2 ml. plasma from portal vessels *30 min.*—2 ml. plasma from portal vessels	321	−30 ± 11.2†

* Mean and standard error.
† Differs significantly from control ($P < 0.01$).

RESULTS

As shown in Table 2, in hydrocortisone-inhibited intact rats the mean change in concentration of adrenal ascorbic acid (mg./100 gm. adrenal weight) following injection of 2 ml. of portal vessel plasma was -6 ± 17.8; whereas, the change for the controls was $+9 \pm 5.4$. When 4 ml. of portal vessel plasma was injected, the change was -30 ± 11.2 and $+6 \pm 4.1$ for the

TABLE 3. EFFECT OF BLOOD PLASMA FROM HYPOTHALAMICO-HYPOPHYSEAL PORTAL VESSELS OF THE DOG ON ADRENAL ASCORBIC ACID OF HYPOPHYSECTOMIZED RATS

No. of rats	Initial treatment	Ascorbic acid concentration, left adrenal (mg./100 gm.) (mean)	Final treatment (blood plasma, i.v.)	Ascorbic acid concentration, right adrenal (mg./100 gm.) (mean)	Difference in ascorbic acid concentration (left adrenal minus right adrenal) (mg./100 gm.) Mean and S.E.
12	Hypophysectomized 22–24 hrs. before use	399	*0 min.*— 2 ml. i.v. plasma from portal vessels *30 min.*—2 ml. i.v. plasma from portal vessels	401	+2 ± 2.8
10	Hypophysectomized 22–24 hrs. before use	465	*0 min.*—2 ml. i.v. plasma from carotid artery *30 min.*—2 ml. i.v. plasma from carotid artery	466	+1 ± 3.9

controls receiving carotid artery plasma. The change in adrenal ascorbic acid concentration in rats receiving 4 ml. of experimental plasma differed significantly from the controls ($P < 0.01$).

Plasma from the portal vessels did not affect the adrenal ascorbic acid concentration in the hypophysectomized rat. As shown in Table 3, the mean change in adrenal ascorbic acid in rats receiving 4 ml. of plasma from the portal vessels and in rats receiving 4 ml. of plasma from the carotid artery did not differ from one another.

DISCUSSION

The release of ACTH in the rat by the anterior lobe can be inhibited by the injection of adrenocortical extracts (Ingle *et al.*, 1937, 1938; Sayers and Sayers, 1945; and Gray and Munson, 1951), cortisone (Sayers and Sayers, 1947 and Abelson and Baron, 1952), and hydrocortisone (Sayers and Sayers, 1947 and Sydnor *et al.*, 1955). Using Long's technic (1955), these observations have been confirmed (Table 1). However, the site of the inhibition effected by these steroids has yet to be determined.

When 4 ml. of plasma from the portal vessels of the dog is injected into the hydrocortisone-inhibited, intact rat, the change in adrenal ascorbic acid concentration measured by the depletion method of Sayers *et al.* (1948) is significantly different ($P < 0.01$) from similarly inhibited rats injected with 4 ml. of plasma from the carotid artery of the dog (Table 2). This response is negated by hypophysectomy (Table 3). These observations are interpreted to mean that the anterior lobe is necessary for the reduction in adrenal ascorbic acid observed in such inhibited, intact rats injected with portal vessel plasma. Furthermore, it would appear that a major block in the response to unilateral adrenalectomy effected by hydrocortisone is at some site other than the anterior lobe, perhaps the hypothalamus, and that the anterior lobe under these conditions can still be stimulated to activity if an exogenous source of portal blood is supplied. These data suggest that venous blood from the hypothalamico-hypophyseal portal vessels contains a substance(s) which accelerates the release of ACTH and that this substance(s) is not present or is present in much less concentration in blood from the carotid artery.

The nature of the active substance is still unknown. It has been suggested by Rothballer (1953), Scharrer and Scharrer (1954), Mirsky *et al.* (1954), and McCann and Brobeck (1954) that the antidiuretic hormone may be a regulating factor in ACTH release. Guillemin has reported (1955a) that ACTH activity in anterior lobe tissue cultures is increased following addition of posterior hypothalamic or eminentia media explants. However, this response was not observed with explants of the anterior hypothalamus. It is of further interest that Guillemin (1955b), using pharmacologic blocking agents, discounts the significance of adrenaline, nor-adrenaline,

acetylcholine, or histamine in the role of specific anterior lobe activators. Elucidation of the nature of the active substance awaits further investigation.

SUMMARY

Plasma from blood obtained from the hypothalamico-hypophyseal portal vessels of hypophysectomized dogs has been used as a possible source of a neurohumoral substance(s) which might influence the rate of release of ACTH by the anterior lobe of the pituitary. Blood plasma from the carotid artery was used for control. Intact rats which had been injected with hydrocortisone acetate in quantity sufficient to prevent adrenal ascorbic acid depletion following unilateral adrenalectomy and hypophysectomized rats were used as test animals.

It was observed that the change in adrenal ascorbic acid concentration in hydrocortisone-inhibited, intact rats following intravenous injection of 4 ml. of portal vessel plasma differed significantly ($P < 0.01$) from similarly treated rats injected with 4 ml. of carotid artery plasma. The change in adrenal ascorbic acid concentration in hypophysectomized rats injected with portal vessel plasma was not different from that observed in hypophysectomized rats injected with plasma from the carotid artery.

These observations suggest that blood from the hypophyseal portal vessels contains a substance(s) which accelerates the release of ACTH from the anterior lobe. These observations further suggest that a block in the response of the rat to the stress of unilateral adrenalectomy occurs at some site other than the anterior lobe, perhaps the hypothalamus.

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EXTRA-THYROIDAL IODIDE METABOLISM IN THE RAT

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THE space of distribution of inorganic iodide in the body is usually considered to be within the confines of the extracellular fluid. There are, however, certain special mechanisms for handling iodide which alter this distribution. Since the iodide compartment or space is of importance in many rate calculations, the following determinations of space of distribution and rate of loss from the blood of radioactive iodine (I^{131}) were made in the rat.[2]

Among the special mechanisms is the ability of the salivary and gastric glands to concentrate and secrete iodide at about 30 times the plasma concentration. Further quantitative information seemed desirable and readily accessible since the salivary and gastric glands simply concentrate iodide and do not bind it to protein and the secretions of these glands can be continuously collected and measured. It was the aim of this study to measure the rate of iodide secretion by the salivary and gastric glands and to study the factors which modify this function.

Renal factors may modify the rate of loss of inorganic iodide from the body and by so doing affect concentration and secretion of iodide by other organs. Attention was directed to the effects of propylthiouracil on the renal excretion of iodide by the report of Wollman and Scow (1955), who found that increasing the dose of propylthiouracil raised the T/S ratio in the mouse by a more rapid fall in blood radioiodine level.

METHODS

Adult male rats fed Purina fox chow were used for these experiments. Propylthiouracil in mildly alkaline solution was injected intraperitoneally. All animals were examined at the end of the study for completeness of thyroidectomy.

For the studies on iodide distribution space the animals were deprived of food and water; 5 mg. of propylthiouracil were followed one hour later by 10 μc. of I^{131}. After allowing one to two hours for equilibration, tail blood specimens of approximately 0.3 ml. were taken at 1 to 2-hour intervals for four to six hours. The radioactivity in 0.1 ml. samples of serum was compared with that of an aliquot of the injected dose. The specimens were diluted to 2.0 ml. and the gamma radiation determined with a suitably shielded scintillation counter.

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For the salivary and gastric studies 250–350 gm. rats were fasted for 18 hours before the experiment. Five milligrams of propylthouracil were given. After 15 to 30 minutes the animals were anesthetized with ether and the abdomen was opened through a mid-line incision. The duodenum was divided 2 cm. distal to the pylorus, the distal end was closed, and the tip of a No. 16 Foley Catheter was inserted into the stomach through the proximal segment. The free margin of proximal duodenum was pulled over and sutured to the thin rubber of the inflatable part of the catheter. The catheter cut to a length of 5 cm. was brought through the abdominal wall via a lateral incision, the abdominal wall was sutured to the thin rubber of the catheter, and the mid-line incision was closed. The stomach was lavaged several times through the catheter, and a small rubber balloon partially expanded was attached to the catheter with a glass adapter. The rat was allowed to recover from the procedure for 1 to 1½ hours and then 10 μc. of I^{131} were injected subcutaneously. The animals were kept in metabolism cages throughout the experiment. At 1 to 2-hour intervals for six to eight hours gastric contents were collected by emptying the balloon and lavaging the stomach with several 2 ml. aliquots of warm water. Urine was collected in the metabolism cages and three 0.3 ml. tail blood specimens were obtained. The gastric samples were liquefied with 4N NaOH and an aliquot of each was counted.

In order to identify the chemical state of the I^{131} secreted into the stomach, the thyroid glands of a group of rats were removed and 5 μg. of L-thyroxin were given daily by intraperitoneal injection, the last dose 24 hours before the experiment. After insertion of the catheter, 50 μc. of I^{131} were injected subcutaneously and a 2 to 3-hour collection of gastric contents was made without gastric lavage. This material was chromatographed directly on Whatman No. 1 filter paper in a butanol-acetic acid-water system. Radioactive iodine was added to gastric juice collected before injection of I^{131} and was run against a specimen collected after injection of I^{131}. In all cases the radioactivity on radioautographs moved with the iodide band.

In the renal studies the thyroid glands were removed from 175–225 gm. rats and they were given 5 μg. of L-thyroxin daily. One week later after fasting overnight the animals were injected with 10 μc. of I^{131}. After 1½ to 2 hours, urination was induced by squeezing the rat and/or by ether inhalation. This urine was discarded. A tail blood sample was obtained for radioactivity determination and the following seven hours' urine was collected. The bladder contents at the end were added to the collection and a second tail blood sample was obtained. Glucose solution (2%) was given as drinking water during the experiment. Animals which did not urinate at the required times were discarded. One week later the study was repeated and the same animals were injected with propylthiouracil after the first blood and urine samples were obtained. One group of rats was fed a diet containing 0.1% propylthiouracil during the week between renal studies. The diet was not otherwise changed. In order to fast this group, propylthiouracil was added to the drinking water the night before the experiment.

RESULTS

Distribution Space of Iodide

In a group of rats injected with 5 mg. of propylthiouracil and followed for four to six hours, the space of distribution of I^{131} was found to be about 50%[3] of the body weight (Table 1). About 11 hours were required for one-

[3] The space of distribution in cc. (V) was calculated by plotting on semilog paper the fraction of injected dose in 1 cc. of serum (I) against time. The straight line so obtained after equilibrium was extrapolated to zero time (I_0). Volume of distribution was taken

TABLE 1. SPACE OF DISTRIBUTION AND HALF-LIFE IN BLOOD OF I^{131} IN ADULT MALE RATS. THE GROUP FROM WHICH THE THYROID GLANDS WERE REMOVED ONE WEEK EARLIER WAS GIVEN THYROXIN 5 μG. DAILY INJECTED INTRAPERITONEALLY. POTASSIUM THIOCYANATE WAS INJECTED INTRAPERITONEALLY 15 TO 20 MINUTES AFTER PROPYLTHIOURACIL

Treatment	No. of animals	Space in % of body weight	Half-life of I^{131} in blood
			(hours)
5 mg. Propylthiouracil	23	48.9 ± 1.4*	11.1 ± 0.9
Thyroidectomy, no Propylthiouracil	14	52.0 ± 2.6	10.4 ± 0.9
5 mg. Propylthiouracil 16 hours before and prior to study	4	51.5 ± 3.2	4.2 ± 0.8
5 mg. Propylthiouracil plus 10 mg. KSCN	7	40.1 ± 1.2	6.4 ± 1.2

* Standard error of mean.

half of the radioactivity to leave the blood. Another group of rats was thyroidectomized one week earlier, and given 5 μg. of L-thyroxin daily by intraperitoneal injection. Propylthiouracil was not given. The space of distribution and rate of loss of radioiodine from the blood did not differ from the first group. Thus the small amount of inorganic iodide in the thyroid gland blocked by propylthiouracil did not alter the space of distribution or rate of loss of iodide from the blood. As a corollary the administration of 5 mg. of propylthiouracil did not alter these values compared to the group not given propylthiouracil.

Propylthiouracil Pretreatment

In a group of rats injected with 5 mg. of propylthiouracil the evening before and again one hour before the radioactive iodine was given, the space of distribution was not altered but the rate of loss of I^{131} from the blood was accelerated to a half-life about 4 hours (Table 1).

Thiocyanate Treatment

The injection of 10 mg. of potassium thiocyanate intraperitoneally 15 to 20 minutes after the propylthiouracil reduced the space of distribution of I^{131} to about 40% of the body weight. The rate of fall in radioactivity from the blood was not significantly altered. In another group of animals the space of distribution and rate of fall of radioiodine concentration in the blood were determined using three blood samples over a 2 to 3-hour period. Immediately after the third blood specimen was obtained, 10 mg. of potassium thiocyanate were injected intraperitoneally. After an interval of $1\frac{1}{2}$ hours three more blood samples were obtained over a three to four-

as $V = I/I_0$. The alternative formulae given by Keating and Albert (1948), and by Riggs (1952) reduce to the one used here when no iodide is held in the thyroid. Intraperitoneal injection of I^{131} in this study reduced the error inherent in this formula due to delay in absorption and distribution.

TABLE 2. EFFECT ON IODIDE SPACE OF ADDITION OF 10 MG. OF KSCN INJECTED INTRAPERITONEALLY AFTER INITIAL SPACE DETERMINATION

Rat No.	Initial space in % body wt. 5 mg. propylthiouracil	Space after KSCN	Change in space as % of body wt.
1	45.8	42.0	− 3.8
2	54.5	40.0	−14.5
3	57.0	41.5	−15.5
4	43.0	34.0	− 9.0
5	61.0	56.0	− 5.0
6	47.3	33.4	−13.9
7	49.5	41.3	− 8.2
8	65.5	49.0	−16.5
	53.0 ± 2.8*	42.2 ± 2.6	10.8 ± 1.7

* Standard error of mean.

hour period. The values for each rat are given in Table 2 and reveal in each instance a decrease in the space of distribution following the administration of thiocyanate. In Table 3 are given the data on the half-life of radioactivity in the blood. In most instances there was no significant decrease in half-life and in 2 animals the rate of fall in the concentration of I^{131} was slower after thiocyanate.

Carrier Iodide

The space of distribution and rate of loss of I^{131} from the blood were not significantly affected by 50 or 200 μg. of carrier added as sodium iodide, nor was the renal clearance of radioiodine (Table 4). Davenport (1943) found little effect on gastric secretion of stable iodide in dogs with gastric pouches until considerably larger amounts of iodide were given.

Combined Salivary and Gastric Secretions of I^{131}

The combined salivary and gastric secretions which accumulated in the stomach contained a total of about 71%[4] of the tracer dose of radioactive iodine (Table 5). It was assumed that there was no significant retention of iodide in the thyroid since the iodide space of distribution was no different whether the thyroid gland was removed or protein binding was blocked with propylthiouracil. The rate of secretion of iodide into the stomach contents was rapid, about one-half of the total being accumulated in the first four hours. The combined salivary and gastric clearance rate was

[4] The method of Keating and Albert (1948), $Q = Qf(1 - e^{-rt})$, has been used to calculate the total per cent of dose of I^{131} secreted by salivary glands and stomach. This assumes that the rate constant of secretion (r) which is common to urine, blood, and thyroid also applies to the salivary and gastric glands (Honour *et al.*, 1952). In this instance (r) was derived from the blood concentrations of I^{131}. The clearance values were calculated according to the method of Albert *et al.* (1952) using the mean plasma I^{131} concentration during each of the 1 to 2 hour gastric collection periods.

TABLE 3. HALF-LIFE OF I^{131} IN THE BLOOD BEFORE AND AFTER KSCN ADMINISTRATION

Rat No.	Initial half-life 5 mg. propylthiouracil only	Half-life after KSCN	Chage
	(hours)	(hours)	(hours)
1	13.3	11.2	−2.1
2	4.7	4.5	−0.2
3	6.4	5.4	−1.0
4	4.6	4.0	−0.6
5	11.7	12.1	+0.4
6	9.7	6.8	−2.9
7	18.3	14.0	−4.3
8	3.4	7.7	+4.3
	9.0±1.8*	7.3±1.4	−0.9±0.9

* Standard error of mean.

0.406 ml. per minute; a rate approximately four to five times the renal clearance of iodide. In the intact animal the iodide is probably rapidly reabsorbed from the small intestine (Albert *et al.*, 1952).

Effect of Removal of Salivary Glands

When the salivary glands were removed as completely as possible about two weeks before the experiment, the total I^{131} collected was about 61% of the dose (Table 5). There was a greater decrease in clearance rate from the untreated group which was lowered 20 to 25%. The contribution of the salivary glands to the total iodide accumulated in the stomach was thus about one-fifth. It was assumed that there was no significant external loss of saliva. The rate constant for secretion into the stomach and for the fall in blood I^{131} was not significantly changed by salivarectomy.

Thiocyanate Administration on Secretion

Intraperitoneal injection of 10 mg. of potassium thiocyanate 15 to 20 minutes after the propylthiouracil (Table 5) resulted in a reduction of total I^{131} in the stomach from 71 to 56%—a reduction of one-fifth—but the

TABLE 4. EFFECTS OF VARIOUS AMOUNTS OF CARRIER IODIDE GIVEN AS NaI WITH I^{131} INTRAPERITONEALLY ONE HOUR AFTER PROPYLTHIOURACIL

	No. of rats	Space as % of body wt.	Half-Life of I^{131} in blood	Renal clearance of iodide in cc./minute
			(hours)	
5 mg. Propylthiouracil	3	50.5	12.1	.090
5 mg. Propylthiouracil 50 μg. Carrier Iodide	2	57.4	14.8	.099
5 mg. Propylthiouracil 200 μg. Carrier Iodide	2	56.2	9.9	.093

TABLE 5. SALIVARY AND GASTRIC SECRETION OF I^{131} MEASURED DIRECTLY WITH A GASTRIC CATHETER. THE VALUES GIVEN FOR PER CENT OF TOTAL DOSE SECRETED ARE THE MEAN OF THE GROUP AFTER 3 TO 4 VALUES EACH REPRESENTING 1 TO 2 HOUR COLLECTIONS FOR EACH ANIMAL ARE AVERAGED. THE RATE CONSTANT OF SECRETION IS A COMMON RATE FOR SECRETION INTO THE STOMACH OR LOSS OF BLOOD CONCENTRATION OF I^{131}

Treatment	No. of rats	% of total dose secreted	Clearance rate of gastric contents in cc./min.	Time in hours for ½ of I^{131} to be secreted	Rate constant of secretion per hour
Propylthiouracil, 5 mg.	5	70.7 ± 2.2*	.406 ± .021	3.8 ± 0.4	.084 ± .006
Salivary glands removed Propylthiouracil, 5 mg.	5	61.0 ± 3.0	.310 ± .030	4.2 ± 0.2	.087 ± .007
Potassium Thiocyanate 10 mg. Propylthiouracil, 5 mg.	3	55.6	.189	6.4	.077
Carrier Iodide, 100 μg. Propylthiouracil, 5 mg.	2	75.3	.363	4.2	.075
L-Thyroxin, 10 μg. daily for 7 days Propylthiouracil, 5 mg.	2	73.3	.390	4.2	.087
Thyroidectomy Thyroxin, 5 μg. daily Thyrotropin, 10 mg.	2	69.9	.237	5.0	.067

* Standard error of mean.

clearance rate was reduced about one-half. This sharp drop in clearance was partially compensated for by the higher blood concentration of I^{131} which did not fall to one-half of its starting level for 6.4 hours. Thus about 6 hours were required for one-half of the I^{131} to be secreted into the stomach in the thiocyanate-treated group compared to only 4 hours in the group not given thiocyanate.

Effect of Carrier Iodide

Addition of 100 μg. of iodide to I^{131} resulted in no significant change in the total gastric and salivary secretion of radioactive iodine, the clearance rate, or the half-time of secretion (Table 5). This amount of iodide is far below the level which affected the ability of the stomach to maintain a concentration gradient for iodide (Davenport, 1943).

Thyrotropin Studies

Two rats were injected with 10 μg. of L-thyroxin daily for 7 days to inhibit endogenous thyrotropin output (McGinty, 1949). The salivary plus gastric secretion of iodide in these animals was compared with the first group given only propylthiouracil and no change was noted. In order further to explore this aspect of the problem rats, after thyroidectomy, were given 5 μg. of L-thyroxin daily. One week later 10 mg. of thyrotropin[5] were

[5] Thyrotropin (Armour) was made up in sterile saline.

injected subcutaneously and beginning 40 hours later (VanderLaan and Greer, 1950), gastric collections of I^{131} were made. The total per cent of the dose secreted was normal but there was a decrease in clearance rate with a slower rate of fall in blood level of I^{131} (Table 5). These findings suggest that the ability of the salivary and gastric glands to secrete iodide was somewhat reduced and there was no evidence of stimulation of iodide secretion by thyrotropin.

Propylthiouracil Effects on Renal Iodide Clearance

The renal clearance of radioiodine[6] after the acute administration of 5 mg. of propylthiouracil per 100 gram body weight was significantly increased when compared with the mean value obtained in the same animals without propylthiouracil (Table 6). In the animals which were given twice

TABLE 6. MEAN RENAL CLEARANCE OF I^{131} IN THYROIDECTOMIZED RATS GIVEN 5 μG. OF L-THYROXIN DAILY. DETERMINATIONS FOLLOWING PROPYLTHIOURACIL ADMINISTRATION WERE COMPARED TO CLEARANCE MEASURED ONE WEEK EARLIER IN SAME ANIMALS WITHOUT PROPYLTHIOURACIL

Propylthiouracil	No. of rats	Initial renal clearance, cc./minute	Clearance after propylthiouracil, cc./minute	Mean of differences for each animal
5 mg. per 100 gm. body weight	8	.085 ± .009*	.132 ± .016	.047 ± .015
10 mg. per 100 gm. body weight	4	.086 ± .015	.167 ± .017	.070 ± .008
0.1% in diet for one week	7	.103 ± .006	.148 ± .029	.040 ± .030

* Standard error of mean.

as much propylthiouracil the mean renal clearance of iodide was almost double the control value. There was a more rapid loss of radioiodine from the blood in both groups. A third group of rats was given a diet containing 0.1% propylthiouracil during the week between the initial and the final study. Based on an estimated intake of 10 grams of food per day these animals received 10 mg. of propylthiouracil daily. The change in mean renal clearance was not statistically significant nor was there a statistically significant change in mean half-life of I^{131} in the blood. Analysis of the mean of the differences in half-life of radioiodine in the blood for each animal, however, gave a minimally significant difference ($P < 0.1$) (Table 7). This method of administration probably resulted in a lesser circulating concentration of propylthiouracil.

Duration of Propylthiouracil Effect

In one group of rats the renal clearance of iodide was measured following the administration of 10 mg. of propylthiouracil per 100 gm. and then re-

[6] The renal clearance values were calculated by the method of Albert *et al.* (1952) using the mean plasma I^{131} concentration over the 7-hour urine collection period.

TABLE 7. MEAN HALF-LIFE OF I^{131} IN BLOOD OF THYROIDECTOMIZED RATS GIVEN 5 μG. L-THYROXIN DAILY. DETERMINATIONS FOLLOWING PROPYLTHIOURACIL WERE COMPARED TO VALUES OBTAINED ONE WEEK EARLIER IN SAME ANIMALS WITHOUT PROPYLTHIOURACIL

Propylthiouracil	No. of rats	Initial mean half-life	Mean half-life after propyl-thiouracil	Mean of differences for each animal
		(hours)	(hours)	(hours)
5 mg. per 100 gm. body weight	8	12.0±1.1*	6.8±0.5	−5.2±1.2
10 mg. per 100 gm. body weight	5	7.5±0.5	5.2±0.9	−2.2±0.6
0.1% in food for one week	7	13.0±2.0	9.6±2.0	−3.3±1.5

* Standard error of mean.

TABLE 8. RENAL CLEARANCE OF IODIDE 4 DAYS AFTER 10 MG. PROPYLTHIOURACIL PER 100 GRAMS BODY WEIGHT COMPARED TO THE VALUES IMMEDIATELY AFTER THE INJECTION

Rat No.	Renal clearance after propylthiouracil, cc./minute	Renal clearance 4 days later, cc./minute	Change in renal clearance, cc./minute
1	.212	.140	−.072
2	.165	.146	−.019
3	.189	.141	−.048
4	.236	.146	−.090
	.201±.015	.143±.002	−.057±.015

measured four days later without propylthiouracil (Table 8). Despite a decrease in clearance rate in all animals, the levels remained substantially higher than any group not previously given propylthiouracil (Table 6).

Effect of Chloride on Iodide Clearance

The renal clearance of radioiodine following the administration of chloride, injected subcutaneously as sodium chloride, was compared with that after 5% glucose solution. Collections of gastric contents were made simultaneously and a total of 2 ml. of glucose or sodium chloride solution were injected as fluid replacement during the 8-hour experiment. In Table 9 the mean clearance values of the group given glucose solution may be compared with those given 1 to 3 m. eq. of chloride. There was a significantly greater iodide clearance when chloride was given.

TABLE 9. RENAL CLEARANCE OF IODIDE (I^{131}) IN RATS GIVEN 5 MG. PROPYLTHIOURACIL AND INJECTED SUBCUTANEOUSLY WITH (Na) CHLORIDE COMPARED TO RATS GIVEN 5% GLUCOSE SOLUTION. THE URINES WERE COLLECTED IN TWO PERIODS DURING AN 8-HOUR EXPERIMENT

	No. of rats	Mean renal clearance, cc./minute
5% Glucose Solution	9	.069±.013
1–3 m.eq. Chloride	7	.195±.045

DISCUSSION

In the rat the space of distribution of inorganic iodide using I^{131} was found to be about 50% of the body weight. This is 10 to 15% higher than the values found in the dog and human being.

Wallace and Brodie (1937) found that the iodide space in the dog varied from 32.8 to 43.4% of the body weight. The large amount of thiocyanate given in their experiment simultaneously with the stable iodide to compare space of distribution of these anions is of the order of magnitude which diminishes gastric and salivary secretion of iodide. In addition the large amount of iodide administered and the blood levels attained were in the range which Davenport (1943) found to reduce the ability of the stomach to maintain a high concentration gradient for iodide. Both of these factors would tend to reduce iodide secretion into the gastrointestinal tract and thus reduce the space of distribution.

In normal human subjects Keating and Albert (1948) using I^{131} found that the inorganic iodide space was equivalent to 36% of body weight and Myant *et al.* (1950) obtained a value of 28% at one hour and 39% at 6 hours after the administration of radioiodine. The difference between these values in man and the larger distribution space in the rat might be explained by the difference in the contribution of the gastrointestinal tract to the total. In the rat the combined salivary and gastric clearance of iodide is about four times the renal clearance whereas Honour *et al.* (1952), found that the total in human beings was only about $1\frac{1}{2}$ times the renal clearance. When 10 mg. of potassium thiocyanate were administered to rats the iodide-distribution space was lowered from 50 to 40% of the body weight, this amount of thiocyanate reducing the clearance rate of iodide into the gastrointestinal tract by one-half. Data given by Honour *et al.* (1952)[7] and by Riggs (1952)[6] indicate a maximum contribution of the gastrointestinal tract to iodide space in man of about 10% of body weight.

A comparison of the combined salivary and gastric secretion of I^{131} to the thyroidal uptake is of interest. Even on a very low iodide diet the uptake of I^{131} by the rat thyroid will not greatly exceed 50% in 6 hours (Astwood, 1955). In these studies 71% of the injected dose was secreted as iodide by the salivary and gastric glands, one-half of the total in the first

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[7] Honour *et al.* (1952) give the mean salivary and gastric clearances of I^{131} as .25 ml. per minute per gram and .12 ml. per minute per gram respectively. These may be combined and compared with the estimate of .83 ml. per minute per gram for the thyroid gland. If the 6-hour uptake of I^{131} by the thyroid is 25%, the 6-hour accumulation in the gastrointestinal tract would be 11% assuming no reabsorption. This figure is probably somewhat overestimated.

Riggs (1952) estimated that in man 3 liters of the total iodide space may be in the stomach based on a value of 100 ml. for the volume of gastric juice and a concentration ratio to blood of 30. If the total space is 25 to 28 liters the gastrointestinal contribution would be 11 to 12%.

four hours. An analysis of the stomach wall for iodide by Wallace and Brodie (1937) revealed levels comparable with those of the kidney, lung, and skin and only 2 or 3 times higher than the liver, spleen, muscle, or pancreas. Thus, just as iodide seems to move rapidly from the thyroid cells into the colloid (Leblond and Gross, 1948) so it is nearly completely secreted into the lumen of the stomach. The effects of thiocyanate and propylthiouracil on salivary and gastric secretion of inorganic iodide found in these observations seem similar to those on the thyroid gland; so also does the effect of carrier iodide. In this study 100 μg. of carrier iodide did not significantly reduce I^{131} secretion into the stomach and Davenport (1943) found that in dogs progressively higher serum iodide concentrations were associated with a progressively greater inability of the stomach to maintain a high concentration gradient for iodide.

In the response to inhibition and administration of thyrotropin, a clear-cut difference seems apparent between salivary and gastric secretion of radioiodine and accumulation of iodide by the thyroid gland, and it is not surprising that these digestive glands seem indifferent to the presence of thyrotropin.

The results of this study are confirmatory of those of Schiff *et al.* (1947) who first used radioiodine to demonstrate the high concentration of iodide in saliva and gastric juice, and of Rowlands *et al.* (1953) who demonstrated the inhibitory influence of potassium perchlorate and potassium thiocyanate on salivary secretion of radioiodine.

The demonstration that propylthiouracil in doses of 5 and 10 mg. per 100 gm. of body weight in the rat increases the renal clearance of iodide has several possible implications. When rats are given a diet containing propylthiouracil it is possible that the increased renal excretion of iodide might contribute to the thyroid enlargement and conversely administration of iodide could correct this effect. If this renal effect of propylthiouracil applies to man as well as the rat, it could be a factor in studies which depend upon blocking protein-binding of iodide by the thyroid and measuring urinary excretion of iodide to calculate the rate of secretion or peripheral degradation of thyroid hormone. Riggs (1952) noted that the excretion of stable iodide during the administration of another member of the thiocarbonamides (1-methyl-2-mercaptoimidazole) was much larger than the daily secretion of hormone estimated by other methods. The rapidity of the increase did not allow increased hormone release as the explanation and it was postulated that extra iodide might leave the thyroid as iodinated tyrosine or as iodide.

Another possible implication of this effect of propylthiouracil is in the mechanism of renal clearance of iodide. To the present no evidence has been found for an active tubular transport mechanism for the reabsorption of iodide from the glomerular filtrate (Riggs, 1952; Bricker and Hlad, 1955). The existence of such a mechanism is suggested by this effect of

propylthiouracil which might act to inhibit the reabsorption of iodide by the renal tubules.

In contrast to man (Bricker and Hlad, 1955) the relation between iodide and chloride secretion by the kidney which Riggs (1949) demonstrated in the dog apparently obtains in the rat as well.

SUMMARY

Inorganic iodide is distributed in a volume equivalent to about 50% of the weight of the rat. Injection of 10 mg. of potassium thiocyanate reduced the iodide space to about 40% apparently by a reduction of secretion of iodide into the gastrointestinal tract. The combined salivary and gastric secretions contained a total of about 70% of an injected dose of radioiodine, the salivary glands contributing about one-fifth of this. Administration or inhibition of secretion of thyrotropin did not appear to affect these values; nor did 100 μg. of carrier iodide. Amounts of carrier iodide up to 200 μg. did not change the distribution space, renal clearance, or rate of loss of I^{131} from the blood.

The renal clearance of iodide was found to be about 0.1 ml. per minute, one-sixth to one-eighth of the filtration rate (Smith, 1951). Administration of propylthiouracil (5 or 10 mg. per 100 gm. of body weight) substantially increased iodide clearance by the kidney. This finding may have implications in studies of thyroid function using propylthiouracil and suggests the presence of an active tubular transport mechanism for iodide.

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Mrs. Raynor Caplan kindly did the iodine determinations.

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THE EFFECT OF DIABETES OF PANCREATECTOMY ON THE PITUITARY CYTOLOGY OF DOGS[1]

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THE pituitary may show characteristic changes after certain "target glands" have been removed. The "thyroidectomy cell" and "castration cell" in the pars distalis of the hypophysis of the rat are two well known examples (Severinghaus, 1938). The present report concerns the changes in the pituitary cytology in dogs with diabetes of pancreatectomy.

METHODS

Fifteen healthy mongrel dogs were pancreatectomized by a standard technic (Markowitz, 1942). All dogs were fed horse meat, pancreatin and brewer's yeast. Blood glucose of each animal was determined daily by the Folin and Malmros method (1929). The resulting diabetes in five dogs (No. 9 and 12–15, Table 1), was treated with a 2:1 mixture of regular and protamine zinc insulin. The remaining ten dogs (No. 1–8, and 10–11, Table 1) received no insulin. All animals were sacrificed by intracardiac injection of choroform after the period of observation. The hypophysis was immediately removed and a careful search for remnants of pancreas was made in each animal. The hypophyses were fixed in Dawson's solution, sectioned at 5μ, and stained with Heidenhain's azan technic (Dawson and Friedgood, 1938). A differential count of the cell types of the pars distalis was made (Rasmussen, 1929), and all slides were examined for abnormal cytology.

OBSERVATIONS

The pertinent data are recorded in Table 1. Severe diabetes was observed in Dogs No. 1–8. In Dogs No. 10 and 11, no persistent hyperglycemia developed. Remnants of pancreatic tissue were found in both animals at autopsy. In dogs No. 12–15, the diabetes following pancreatectomy was controlled with insulin. The diabetes was not well regulated with insulin in Dog. No. 9. In all the uncontrolled or poorly controlled Dogs, (No. 1–9) the pars distalis of the hypophysis exhibited several cytological changes when compared with those from normal dogs. Many of the basophile cells in the pars distalis of the hypophyses of Dogs No. 1–9 (Figs. 1 and 2) were found to have a diameter four to six times that of the basophile cells

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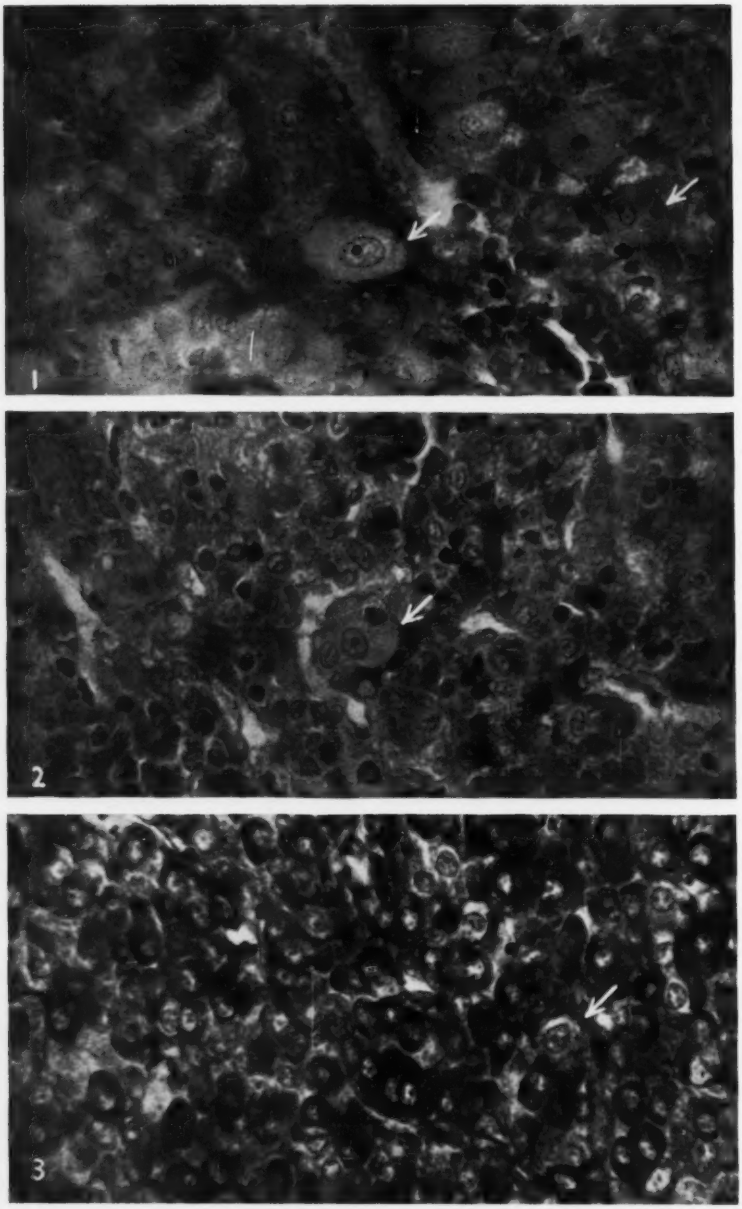

FIG. 1. Pars distalis of the hypophysis of a dog with uncontrolled diabetes of pancreatectomy (Dog No. 4). Arrows indicate enlarged, degranulated and vacuolated basophile cell. Heidenhains azan. ×670.

FIG. 2. Pars distalis of the hypophysis of a dog with uncontrolled diabetes of pancreatectomy (Dog No. 3). Arrow indicates an enlarged and degranulated basophile cell. Heidenhains azan. ×670

FIG. 3. Pars distalis of hypophysis of a normal dog. Arrow indicates a normal basophile cell. Heidenhains azan. ×670.

of normal dogs (Fig. 3). These enlarged basophile cells were partially or totally degranulated, or had granules too small to be resolved with the ordinary microscope. When the cells contained no granules, their basophilic origin was indicated by their characteristic blue cytoplasm. Vacuolization could occasionally be seen in the basophilic cytoplasm. Acidophile and chromophobe cells in the pars distalis of the hypophyses of these animals were identical with those seen in normal dogs.

There was no enlargement or degranulation of the basophile cells of the pars distalis in the hypophyses of Dogs No. 12–15, in which the diabetes of

TABLE 2. THE EFFECT OF PANCREATECTOMY DIABETES ON PITUITARY CYTOLOGY

Dog No.	Sex	Life span (days) following pancreatectomy	Blood glucose level following pancreatectomy			Basophilic change	Pituitary cell count %		
			Range	Avg.	Level just before death		Baso.	Acid.	Chromophobe
1	M	7	280	—	280	+*	7.65	55.21	37.14
2	M	8	338–372	355	338	++	9.01	45.59	45.40
3	F	7	338–365	350	346	++	4.83	50.71	44.46
4	M	14	213–300	250	213	+++	5.51	53.67	40.82
5	M	5	463–775	572	775	+++	4.83	50.71	44.46
6	M	16	274–431	329	431	+++	7.74	62.97	29.29
7	M	25	292–520	433	413	++++	7.83	45.62	46.55
8	M	37	308–422	358	385	++++	5.10	59.83	35.07
9	M	21	33–520	323	481	+++	5.14	44.70	50.16
10	M	17	72–115	88	115	0	6.55	51.21	42.24
11	M	12	93–303	146	102	0	7.93	57.71	34.36
12	F	27	50–340	164	90	0	8.14	53.23	38.63
13	F	27	59–267	142	86	0	5.20	57.13	37.67
14	F	49	51–355	143	92	0	5.49	37.44	57.07
15	M	36	62–390	130	120	0	2.96	45.79	51.25

* Denotes relative degree of the qualitative changes described in the text.
0—Denotes normal basophile cells.
Dog 9—Insulin given but diabetes poorly controlled.
Dogs 10 and 11—Remnants of pancreatic tissue found at autopsy.
Dogs 12, 13, 14 and 15—Diabetes controlled with insulin.

pancreatectomy was controlled with insulin. The acidophile and chromophobe cells in these animals were normal. The cells of the pars distalis of the hypophyses were normal in Dogs No. 10 and 11, in which persistent hyperglycemia did not develop and in which remnants of pancreas were found at autopsy.

The differential cell counts of the pars distalis of the hypophyses of all dogs revealed no quantitative change. As can be seen from Table 1, our counts were within the range of counts that have been made by other investigators on normal dogs (White and Faust, 1944; Francis and Mulligan, 1949; Goldberg and Chaikoff, 1952).

DISCUSSION

Definite qualitative changes in the basophile cells of the pars distalis of the hypophysis were demonstrated in dogs with uncontrolled or poorly controlled diabetes of pancreatectomy. The severity of the basophilic changes appeared to depend on the duration of the diabetes rather than on the degree of hyperglycemia. When the diabetes was controlled with insulin or failed to develop because of remnants of pancreas, the changes in the basophiles did not occur. This suggests that the changes in the

basophile cells can be prevented or reversed by insulin, and that the changes cannot be attributed to the procedure of pancreatectomy *per se*. The precise mechanism involved in the causation of the qualitative changes in the basophile cells of the hypophyses due to insulin deficiency has not been elucidated by this experiment. To our knowledge, there has been no report of a cytological study of the pars distalis of the hypophysis in dogs or other animals with diabetes of pancreatectomy. Thomas and Emerson (1945) found degranulation, hyalinization and even pyknosis or complete destruction of the basophiles in the pars distalis of the hypophyses of rabbits and rats following the administration of massive doses of alloxan. Although Warren and LeCompte (1952) have concluded that no constant and significant changes occur in the hypophyses of patients with diabetes mellitus, observations on the pituitary cytology in human diabetes associated with pancreatic destruction seem lacking.

SUMMARY

Basophile cell changes in the form of enlargement, degranulation, and vacuolization were observed in the hypophyses of dogs with diabetes of pancreatectomy. These changes were not demonstrated if the diabetes was controlled with insulin or if the presence of remnant pancreatic tissue prevented the development of diabetes.

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HORMONAL CONTROL OF GLYCOGEN IN THE HEART AND OTHER TISSUES IN RATS[1]

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THE glycogen of the heart, unlike that of other tissues, has often been found to be greater in amount in the fasting animal than in the fully fed subject (Macleod and Prendergest, 1921; Evans, 1934; Bong, Junkersdorf and Steinborn, 1934; Lackey, Bunde, and Harris, 1946). Illingworth and Russell (1951) observed further that the cardiac glycogen of fasting hypophysectomized rats was lower than that of normal animals similarly fasted and that this difference could be overcome to a considerable degree by treatment with purified growth hormone during the fasting period. Also the cardiac glycogen of intact fasting rats was increased after the acute administration of growth hormone. These observations suggested that the activity of one or more pituitary hormones might be concerned with the increase in cardiac glycogen which is induced by fasting, and the experiments below were undertaken primarily as a further investigation of this point. In the same animals, glycogen was determined also in the gastrocnemius muscle, diaphragm, and liver; and in addition, the opportunity was taken to observe whether the glycogen of the heart and diaphragm, like that of the leg muscle and liver, could be separated into trichloroacetic acid extractable and non-extractable portions as described by Bloom, Lewis, Schumpert, and Shen (1951), and if so whether these fractions were affected differentially by the feeding and fasting regimens employed here or by treatment with growth hormone.

The data obtained indicate that growth hormone is necessary for the increase in cardiac glycogen to occur during fasting in the rat. It was found also that the extractable glycogen was more variable physiologically than the non-extractable fraction in all the tissues examined.

Portions of the material presented here have been discussed in a preliminary communication (Russell, 1953).

METHODS

The animals employed were male rats of the Sprague-Dawley strain, in most cases 3 to 4 months of age and weighing 250–300 gm. They were maintained at 24–26° C.

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and fed a high carbohydrate diet (Purina Laboratory Chow). For analyses of tissue constituents in the unfasted state, the animals were taken from their cages at about 2 P.M. and sacrificed shortly thereafter. Fasting periods of 24 hours were begun and ended also at about 2 P.M. The rats fed glucose were first fasted overnight (18–20 hrs.) and then given 5 ml. of 30% (w/v) glucose by stomach tube 4 hours before sacrifice. Usually the tissues of 4 animals were taken for analysis at one time, with each group of concurrent subjects including representatives from two or more experimental series.

The hypophysectomized or adrenalectomized rats were prepared by the usual techniques 2–4 weeks before experiment and were inspected at autopsy for completeness of the operative removal of the respective organs. The adrenalectomized rats were maintained with salt (0.7% NaCl, 0.3% $NaHCO_3$) in place of drinking water after operation.

Two preparations of growth hormone were employed. That given to the fasting hypophysectomized rats was prepared in this laboratory by the method of Wilhelmi, Fishman, and Russell (1948). It was electrophoretically homogeneous, contained a very low order of contamination by other pituitary hormones, and was highly active in promoting growth in hypophysectomized rats. In the present experiments it was given in a total dose of 0.5 mg./100 gm. body weight, each dose divided into 3 equal portions and injected intraperitoneally at the start of the fasting period, 5–7 hours later, and again 6 hours before the tissues were taken, respectively. The growth hormone preparation given to the intact animals was from lot no. 22KR-2 prepared by the Armour Laboratories according to the same method. It contained some thyrotrophic hormone but little adrenocorticotrophin, and at the time it was employed for these experiments it was slightly less active in growth promotion tests than was the former preparation.

Insulin, regular, was given intraperitoneally in a dose of 5 units per kg. body weight immediately after the glucose was fed.

The tissue samples were taken from the animals under pentobarbital anesthesia (6 mg./100 gm. for the intact animals, 4 mg./100 gm. for the operated rats) in the following order: both gastrocnemii (reported as a single average value), the heart, diaphragm, and liver. The heart was taken as quickly as possible after opening the chest in order to minimize the rapid glycogenolysis which occurs in this organ when it continues to beat without adequate oxygenation. The diaphragm was necessarily removed after the heart, and so some glycogenolysis may have occurred in this tissue. Immediately on removal, each tissue sample was compressed forcibly between two blocks of solid carbon dioxide. The thin frozen cakes of tissues so obtained were then trimmed free of obvious connective tissue and blood (most of the blood in the heart is expressed during the freezing operation), dissected, and weighed while still frozen. In each case, the heart, leg muscle, and diaphragm were broken into several smaller fragments, of which half were placed in alkali and digested for estimation of total glycogen and half were placed in 10% trichloroacetic acid for extraction of the "free" glycogen as described by Bloom *et al.* (1951) and Russell and Bloom (1955). Liver samples weighing two grams or more were placed in a well in the cake of solid carbon dioxide, ground to a fine powder, and mixed thoroughly before aliquots of about 0.5 gm. each were taken for parallel analyses in the same way. In all cases the glycogen was precipitated with two volumes of ethanol, allowed to stand overnight in the cold, collected by centrifugation, and finally estimated by the anthrone reaction as described by Morris (1948). The residual or "bound" glycogen was obtained by difference between total and extractable glycogen in each case. All glycogen concentrations are expressed as weight of glucose in the wet tissue.

RESULTS

1. *Cardiac glycogen.* In the first series of experiments, the glycogen content of tissues from normal, hypophysectomized, or adrenalectomized rats

was determined in animals which were either in the unfasted state or which had been fasted for 24 hours. As shown in Table 1, parts A and B, the glycogen content of the hearts of fed hypophysectomized rats was normal or slightly elevated. During fasting the cardiac glycogen of the normals rats underwent a marked increase, the total glycogen being augmented by about 50% and the extractable glycogen nearly doubled. In the absence of the hypophysis, this increase in glycogen of the heart did not occur; but if growth hormone was given to the operated animals during the fasting

TABLE 1. TOTAL, TRICHLOROACETIC-ACID EXTRACTABLE, AND RESIDUAL GLYCOGEN IN THE HEART OF RATS

	No. of observ.	Glycogen—mg. per cent[4]		
		Total	Extractable	Residual
A. Unfasted				
1. Normal	11	408 ± 15	203 ± 9	205 ± 12
2. Adrenalectomized	8	396 ± 17	192 ± 19	203 ± 11
3. Hypophysectomized	7	468 ± 19*	242 ± 12*	226 ± 16
B. Fasted 24 hrs.				
1. Normal	12	615 ± 12	382 ± 8	233 ± 13
2. Normal + G.H.[1]	8	695 ± 17**	443 ± 26*	252 ± 21
3. Adrenalectomized	6	572 ± 21	361 ± 23	210 ± 13
4. Hypophysectomized	6	402 ± 14**	167 ± 17**	235 ± 14
5. Hypophysect. + G.H.[2]	9	616 ± 24	370 ± 20	246 ± 16
C. Fed Glucose (4 hrs.)				
1. Normal	10	531 ± 33‡	287 ± 23‡	244 ± 18
2. Normal + G.H.[1]	9	782 ± 41**	557 ± 42**	225 ± 15
3. Normal + Insulin[3]	7	643 ± 24*	404 ± 21**	239 ± 19

[1] Growth hormone, 1 mg./100 gm. i.p. 6 hrs. before sampling.
[2] Growth hormone, 0.5 mg./100 gm. i.p. in 3 doses over 24 hrs.
[3] Insulin, 5 U./kg., i.p. 4 hrs. before sampling.
[4] With standard error of the mean.
* $p < 0.02$.
** $p < 0.01$ for difference from respective normal group.
‡ $p < 0.02$ for difference from fasting normal group.

period, the normal accumulation of glycogen was seen. As shown also in Table 1, the cardiac glycogen of adrenalectomized rats did not differ from the normal either in the fed or fasted state. Hence it appears that the increase in glycogen of the heart which occurs during fasting is determined mainly by the presence of the pituitary growth hormone or a closely related factor.

In another group of this series, a single injection of growth hormone was given to normal fasted rats 6 hours before analysis (Table 1, B). In confirmation of earlier observations (Illingworth and Russell, 1951), the cardiac glycogen was increased significantly; and this increase occurred entirely in the extractable fraction of the glycogen.

In a second series of experiments, normal rats were first fasted and then fed glucose (about 0.5 gm./100 gm. body weight) with or without the prior injection of growth hormone or of insulin. Four hours after the glucose feeding alone, the cardiac glycogen had fallen from the high level characteristic of the fasting state to a point about midway between the latter level and that seen in the fully fed state (Table 1, C). A smaller but significant

reduction in cardiac glycogen after glucose was seen also by Evans (1934). In contrast, in animals given growth hormone 2 hours before the glucose feeding, the glycogen content of the heart not only did not fall in this way but instead rose considerably above the level seen in the untreated fasting animals. Furthermore, the average value for the cardiac glycogen of the hormone treated, glucose fed animals (group C-2) exceeded also that of the fasting animals given growth hormone alone 6 hours before analysis (group B-2); but because the figures obtained in the former series were rather variable, this difference was of marginal significance (for total glycogen, $t = 1.9$, $p = 0.1$–0.05; for extractable glycogen, $t = 2.2$, $p = 0.05$). However, the very high concentrations of glycogen seen in the hearts of some of these animals—as high as 1% glycogen, exceeding all values obtained in any other condition—suggest sthat the growth hormone may have been more effective in promoting glycogen deposition in the heart when glucose was given than when it was not. In any case, it is evident that a single injection of growth hormone enabled the maintenance of very high concentrations of glycogen in the heart even in the presence of a plethora of glucose.

The fall in cardiac glycogen which otherwise occurred when glucose was fed was prevented also when a rather large dose of insulin (5 units per kg.) was given along with the glucose (group C-3); but no abnormally high concentrations of glycogen were seen in this series. Since this large dose of insulin could not be given alone to fasting animals, it could not be determined whether this amount of insulin would augment the cardiac glycogen in the absence of administered glucose. In previous experiments, a small amount of insulin, sufficient to induce hypoglycemia comparable to that observed after growth hormone alone, had no effect on the cardiac glycogen of fasting animals (Illingworth and Russell, 1951). An intermediate quantity, 1 unit per kg., tended to bring about an increase in the fasting cardiac glycogen, but this was most probably an indirect result of epinephrine secretion invoked by hypoglycemia (Bloom and Russell, 1955). It seems likely then that the insulin by itself would have had little effect and that the maintenance of cardiac glycogen observed here when insulin was given to glucose fed animals represented deposition of glycogen in the usual manner. Compared to other muscles, however, the heart appeared to be relatively insensitive to the glycogenic action of the insulin.

As may be seen in Table 1, nearly all of the alterations induced in the glycogen content of the heart in these experiments occurred in the trichloroacetic acid extractable portion. Minor variations were seen in the residual or non-extractable fraction, but in no case was there a significant difference between the average value found for this moiety in an experimental group and the corresponding value in the appropriate control group. Furthermore, the residual glycogen, in spite of having been determined by difference, appeared to be less variable within groups than either

the total or extractable glycogen; the standard deviations of the residual groups were usually smaller than those for the corresponding total glycogen values, and the composite estimate of the error variance for the residual series was significantly less than that for either the total or extractable glycogen (variance ratios, 2.27 and 1.91 respectively, 82 d.f., $p < 0.01$ in each case). Thus, within groups, as well as between groups, the extractable glycogen appeared to be the principal variable moiety in the heart in these conditions.

In the dog heart, Merrick and Meyer (1954) observed a similar relative degree of lability of the extractable glycogen as compared to the bound glycogen during post-mortem glycogenolysis. During tamponade with anoxia induced by forced pneumothorax, however, these investigators reported that the residual fraction increased as the extractable portion fell. No evidence of any such shift in the proportions of the two fractions has appeared in this or other investigations on the rat heart (Russell and Bloom, 1955; Bloom and Russell, 1955). The data of Merrick and Meyer on the rat heart are not comparable to those presented here because of the large losses of cardiac glycogen incurred by their method of sacrifice (Russell and Bloom, 1955).

2. *Gastrocnemius glycogen.* The principal interest in these data and also in that from diaphragm attaches to the comparisons to be made between the effects seen here with those occurring in the cardiac glycogen in the same animals, and in the relative changes occurring in the extractable and non-extractable glycogen fractions. In normal and in adrenalectomized rats, the glycogen of the gastrocnemius remained unchanged during fasting in these experiments, whereas the cardiac glycogen had increased considerably. On the other hand, in hypophysectomized rats the glycogen of both organs fell during fasting. When growth hormone was given to the latter animals, the gastrocnemius glycogen was maintained at a slightly subnormal level, where that of the heart was increased as it was in the normal fasting subject. In the normal fasting rat, a single dose of growth hormone had no effect on the gastrocnemius glycogen, although the cardiac glycogen of the same animals was increased significantly. Similarly in the glucose fed rat, growth hormone had but little effect on deposition of glycogen in the leg muscle in these experiments (no significant increase in total glycogen, but probably an increase in the extractable fraction); whereas a striking difference was observed between the levels of cardiac glycogen found in the untreated and in growth hormone treated animals. Insulin, on the other hand, was probably somewhat more effective than growth hormone in respect to glycogen deposition in gastrocnemius muscle, although it had had less effect on the heart. Hence, the gastrocnemius glycogen appears to be considerably less sensitive to the action of growth hormone than is that of the heart, but it is perhaps more sensitive to insulin.

In a summary of data on the relative proportions of extractable and

non-extractable glycogen in the tissues of rats in a wide variety of conditions, Russell and Bloom (1955) reported that in the gastrocnemius muscle the quantity of the extractable glycogen was correlated closely with the total glycogen, whereas the non-extractable fraction exhibited generally but a small degree of increase with increasing total or extractable glycogen. The group means of Table 2 conform for the most part to this relationship. For example, where fasting hypophysectomized animals with and without growth hormone are compared with fasting normal rats, the loss of glycogen and its replacement appear to have taken place largely, but not quite

TABLE 2. GLYCOGEN IN THE GASTROCNEMIUS MUSCLE OF RATS[1]

	No. of observ.	Glycogen—mg. per cent		
		Total	Extractable	Residual
A. Unfasted				
1. Normal	11	502 ± 18	258 ± 11	244 ± 18
2. Adrenalectomized	8	533 ± 21	258 ± 21	275 ± 20
3. Hypophysectomized	7	591 ± 20**	264 ± 12	327 ± 9**
B. Fasted 24 hrs.				
1. Normal	10	525 ± 11	251 ± 16	274 ± 14
2. Normal + G.H.	9	522 ± 20	222 ± 18	301 ± 13
3. Adrenalectomized	7	500 ± 23	249 ± 11	251 ± 17
4. Hypophysectomized	6	351 ± 7**	117 ± 7**	234 ± 6*
5. Hypophysect. + G.H. during fast	9	485 ± 13*	193 ± 6**	292 ± 12
C. Fed Glucose 4 hrs.				
1. Normal	10	672 ± 19	340 ± 15	332 ± 14
2. Normal + G.H.	10	691 ± 28	392 ± 20*	300 ± 12
3. Normal + Insulin	7	752 ± 20**	428 ± 17**	324 ± 17

[1] See Table 1 for details of treatment.
* $p < 0.05$.
** $p < 0.01$ for difference from respective normal group.

entirely, in the extractable fraction. Also, in the several series in which the total glycogen was much increased by the feeding of glucose, both residual and extractable fractions were increased, but the further increments induced by treatment with growth hormone or insulin took place entirely in the extractable moiety. Thus, with one possible exception, there does not appear to be any specific effect of any of the treatments employed here on the non-extractable glycogen. The unfasted hypophysectomized rats did show exceptionally high levels of residual glycogen in the gastrocnemius msucle. Since there is no obvious explanation for this discrepancy. further confirmation of these data is necessary.

Within groups, as well as between groups, the total glycogen tended to be the most variable and the residual glycogen the least variable quantity. With 83 degrees of freedom, the ratios of the composite estimates of the internal variance were for total vs. residual glycogen 2.04, $p < 0.01$; for extractable vs. residual glycogen, 1.32, $p = 0.1$–0.05; and for total vs. extractable, 1.55, $p < 0.05$.

3. *Diaphragm glycogen.* As shown in Table 3, the alterations seen in the glycogen content of the diaphragm in these experiments were for the most

part qualitatively similar to those occurring in the gastrocnemius muscle, but they were generally greater in magnitude. Normal rats, not fasted, maintained an average glycogen concentration of about 0.4%, a value rather lower than that observed in other muscles such as the gastrocnemius. In adrenalectomized and in hypophysectomized rats in the unfasted state, the diaphragm glycogen was significantly higher than in the normal animals. This difference may have arisen because both types of operated animals tend to eat more frequently throughout the day than do normal rats, and so at the time of sacrifice (early afternoon) they may have taken

TABLE 3. GLYCOGEN IN THE DIAPHRAGM IN RATS[1]

	No. of observ.	Glycogen—mg. per cent		
		Total	Extractable	Residual
A. Unfasted				
1. Normal	11	371 ± 26	155 ± 15	216 ± 15
2. Adrenalectomized	8	520 ± 19**	244 ± 12**	276 ± 14**
3. Hypophysectomized	7	546 ± 36**	228 ± 24*	319 ± 25**
B. Fasted 24 hrs.				
1. Normal	11	297 ± 14‡	128 ± 10	169 ± 12‡
2. Normal + G.H.	8	294 ± 15	106 ± 11	188 ± 18
3. Adrenalectomized	6	339 ± 28	131 ± 10	208 ± 20
4. Hypophysectomized	5	180 ± 20*	53 ± 8*	138 ± 21
5. Hypophysect. + G.H. during fast		302 ± 19	107 ± 7	195 ± 17
C. Fed Glucose 4 hrs.				
1. Normal	7	953 ± 89	582 ± 84	371 ± 25
2. Normal + G.H.	10	1146 ± 65	788 ± 45	358 ± 42
3. Normal + Insulin	6	1327 ± 146*	882 ± 94*	445 ± 67

[1] See Table 1 for details of treatment.
* $p < 0.05$ for difference from respective normal group.
** $p < 0.01$ for difference from respective normal group.
‡ $p < .02$ for difference from unfasted normal group.

food more recently. During fasting the diaphragm glycogen of the normal and of the adrenalectomized rats fell to about the same level; but, as reported previously (Illingworth and Russell, 1951), that of the hypophysectomized rats was reduced much further, and this fall was prevented by treatment with growth hormone during the fast.

The remaining observations on this tissue are confirmatory of previous reports and indicate the very great deposition of glycogen which occurs in the diaphragm after the feeding of glucose and the further increase in this deposition which may be obtained by treatment with either growth hormone or insulin.

The greater part of the changes in diaphragm glycogen in these experiments appeared also to occur in the trichloroacetic acid extractable fraction. As reported elsewhere (Russell and Bloom, 1955), the residual fraction is more variable in this tissue than it is in the heart or gastrocnemius, but with increasing quantities of glycogen the alterations in this fraction are always much less than in the extractable portion. In the data of Table 3, also, it may be seen that where the total glycogen was increased

above the normal fasting level, both fractions were significantly augmented. However, in the glucose fed animals where the extractable fraction was increased four-fold or more, the bound glycogen was only doubled; and where further increases in the extractable glycogen occurred as the result of treatment, no significant changes were seen in the residual fraction. Among the fasting animals, also, there were no alterations in the residual glycogen, the fall in glycogen characteristic of the hypophysectomized rat occurring to a significant degree only in the extractable portion. Thus in the diaphragm as in the heart and gastrocnemius muscle, the extractable moiety appeared to be the principal physiological variable.

4. *Liver glycogen.* The figures for total glycogen concentrations in the liver, presented in Table 4, are those to be expected of animals in the experimental conditions employed. In the livers from animals which were unfasted or fed glucose, in which the total glycogen was above 1.5%, the trichloroacetic acid extractable glycogen was closely correlated in amount with the total glycogen, whereas the residual or bound glycogen remained constant at approximately 0.5%. Among the six groups in this category, analysis of variance indicated no significant differences in the quantities of bound glycogen, although there were some differences between groups in amount of extractable glycogen. The mean proportions of extractable glycogen then varied between 75 and 85% of the total. In the livers of fasting animals, on the other hand, where the extractable glycogen declined to extremely low levels, the residual fraction remained always the greater in amount. Here, the mean proportion of extractable glycogen ranged from near zero to at most about 40%. Thus, although the residual glycogen was by no means invariable in the liver, the trichloroacetic acid extractable

TABLE 4. GLYCOGEN IN THE LIVER OF RATS[1]

	No. of observ.	Glycogen—per cent		
		Total	Extractable	Residual
A. Unfasted				
1. Normal	11	2.84 ± .22	2.38 ± .20	0.46 ± .09
2. Adrenalectomized	8	2.18 ± .46	1.73 ± .45	0.45 ± .09
3. Hypophysectomized	7	2.58 ± .60	1.98 ± .54	0.60 ± .08
B. Fasted 24 hrs.				
1. Normal	12	0.13 ± .01	0.03 ± .01	0.10 ± .01
2. Normal + G.H.	8	0.39 ± .09	0.17 ± .06	0.22 ± .04
3. Adrenalectomized	7	0.10 ± .03	0.03 ± .02	0.07 ± .02
4. Hypophysectomized	6	0.06 ± .002	0.001	0.06 ± .002
5. Hypophysect. + G.H. during fast	9	0.07 ± .004	0.003	0.07 ± .003
C. Fed Glucose, 4 hrs.				
1. Normal	10	3.30 ± .15*	2.65 ± .17†	0.66 ± .09‡
2. Normal + G.H.	10	2.82 ± .10*	2.25 ± .10†	0.57 ± .05‡
3. Normal ± Insulin	7	2.35 ± .18*	1.98 ± .18†	0.35 ± .09‡

[1] See Table 1 for details of treatment.
* For difference among these groups, $F = 10.3$, $p < .001$.
† For difference among these groups, $F = 4.7$, $p < .05$.
‡ For difference among these groups, $F = 3.1$, $p > .05$.

glycogen fraction displayed much the greater range of variation. Beyond this relationship, no specific effects of treatment on the quantities or proportions of the two fractions could be identified in this work.

DISCUSSION

From the observations presented here on the cardiac glycogen of the rat, it appears that in this species the pituitary gland is necessary for the normal accumulation of glycogen to take place during fasting, that the presence of the adrenal gland is not required, and that replacement of the pituitary in this response to fasting can be brought about by treatment with purified growth hormone. Furthermore, the effect of feeding carbohydrate to the fasting animal, which results in a prompt fall in the cardiac glycogen, is more than overcome by a single injection of growth hormone. Also, as shown previously, a further increase in the fasting level of cardiac glycogen in the intact animal can be induced by growth hormone. These observations suggest that the amount of glycogen in the rat heart may serve to some extent as an indicator of the activity of growth hormone or of a closely related pituitary factor, and, as a further working hypothesis, that the secretion of this hormone is normally increased during fasting and inhibited by the presence of carbohydrate in quantity. It is possible that the activity of the hormone, rather than its rate of secretion, would be affected in these circumstances, but the latter seems more probable since the greatest increase in cardiac glycogen was seen when growth hormone and glucose were given together. Such regulation of growth hormone secretion or activity by the nutritive state would be consonant with other known effects of the hormone; for two important adaptive responses to fasting—the conservation of nitrogen and the replacement of carbohydrate by fat in the metabolic mixture—are known to be defective in hypophysectomized animals and to be favored by pituitary growth hormone preparations. More detailed observations on dosage-response curves and on the time relationships of these effects are of course required to substantiate these suggestions. A preliminary report of some additional work of this nature has been presented by Adrouny and Russell (1954).

Deposition of glycogen under the influence of insulin, a characteristic effect of this hormone in skeletal muscle, does not seem to be strongly manifest in the heart. In the present work, a large dose of insulin given with carbohydrate induced much extra deposition of glycogen in the gastrocnemius and especially in the diaphragm, but in the heart it was able only to prevent the fall in glycogen which otherwise occurred after glucose alone. Smaller doses of insulin have not generally been observed to affect the cardiac glycogen *in vivo*. Hence, the effect of growth hormone seen here cannot have been mediated by the secretion of insulin. Further evidence of the relative independence of the cardiac glycogen from the supply of insulin is seen in the high glycogen levels often reported in diabetic animals

(Cruickshank, 1913; Banting, Best, Collip, and Noble, 1922; Fisher and Lackey, 1925; Cruickshank and Shrivastava, 1930; Evans and Bowie, 1936; Lackey, Bunde, Gill and Harris, 1944; Illingworth and Russell, 1951). This has been thought possibly to be related to the high blood glucose levels prevailing in these animals, but the fact that the glycogen of the heart of normal animals is depressed by glucose feeding argues against this view. It is conceivable that the increased content of glycogen in the hearts of diabetic animals is an expression of increased pituitary activity in this condition, but further observations are necessary to establish this point.

The mechanism by which growth hormone brings about the increase in glycogen of the heart is not apparent. It could be due to increased uptake of glucose from the blood, to inhibition of the further utilization of stored glycogen being formed from the blood glucose at a normal rate, or possibly to increased formation of glycogen from non-carbohydrate precursors within the heart. In calling attention to a degree of correlation between the cardiac glycogen and blood ketone levels in fasting and fed rats, Lackey, Bunde and Harris (1946) suggested the possibility of conversion of ketone bodies to glycogen in the heart. Since there is no direct evidence that such a conversion can occur, it is rather more probable that the ketosis of fasting, or that seen after growth hormone, and the increases in cardiac glycogen are parallel expressions of the metabolic alterations which occur in these conditions. The well-known anabolic action of growth hormone contraindicates any increase in gluconeogenesis from amino acids, although of course the possibility of specific effects upon the heart cannot be excluded; nor are there any indications that the supply of lactic acid as a precursor of glycogen would be increased in these conditions. Hence, synthesis of new glycogen from non-carbohydrate sources does not seem a likely explanation of these effects. With respect to the first two alternatives suggested above, available evidence does not yet permit a decision. However, there has been little evidence presented to suggest that growth hormone increases the uptake of glucose by other tissues; but there are many observations interpretable as indicating a diminution in carbohydrate utilization. If, therefore, the action of the growth hormone on the heart is similar to that in other tissues, inhibition of glycogen utilization would appear to be the most probable mechanism by which the cardiac glycogen is augmented by growth hormone and also presumably by fasting. Attempts by one of the authors (JAR) to observe further the action of growth hormone on heart slices *in vitro* have so far been unsuccessful, because most of the carbohydrate in the slices has been found to be lost into the medium within a few minutes of the start of incubation.

Comparison of amounts of glycogen extractable with trichloroacetic acid with those bound to protein (the residual fraction) in the heart and other

tissues in the varying conditions of these experiments indicates that for the most part the extractable glycogen was the more variable fraction physiologically. This is particularly true of the heart, where the bound glycogen remained practically constant throughout these studies. In the other muscles observed, this fraction did undergo some significant alterations. These were in nearly every case in the same direction as the changes in total and in extractable glycogen, but they were usually much smaller in magnitude. In the livers of fed, animals where the glycogen content was fairly high, the residual glycogen remained essentially constant also, and in fasting animals, the depletion of glycogen in this fraction was relatively less extensive than was that of the extractable glycogen. These results do not indicate any specific effects of any of the procedures employed here on the ratios of free to bound glycogen, nor do they suggest any conversion of one fraction to another. Rather, they suggest that both fractions tend to vary somewhat in the same manner, but that in general the extractable fraction is much the more labile moiety.

SUMMARY

Where the glycogen of the heart of normal or of adrenalectomized rats was increased by about 50% during a 24-hour fasting period, that of hypophysectomized rats fell somewhat during this time. The administration of purified growth hormone to hypophysectomized rats during the fasting period permitted the occurrence of the normal increase in cardiac glycogen. In normal fasting rats, the glycogen of the heart was significantly reduced 4 hours after the feeding of glucose in quantity. Growth hormone given to these animals 2 hours before the glucose feeding not only prevented this reduction but instead induced marked deposition of glycogen in the heart. Insulin in large dosage given with the glucose prevented the fall in cardiac glycogen but did not bring about any further increase. These observations are taken to suggest that the level of glycogen in the rat heart may serve to some extent as an indicator of growth hormone activity, and also possibly to indicate that either the rate of secretion or the activity of growth hormone (or of a closely related pituitary factor) is normally increased during fasting and suppressed by carbohydrate in quantity.

In the same animals, alterations in the glycogen of the gastrocnemius muscle and of the liver were similar to those previously reported in these circumstances. The effects on the glycogen of the diaphragm resembled qualitatively those in the gastrocnemius but were generally greater in magnitude. The glycogen of both of these muscles appeared to be less sensitive to the action of growth hormone than was that of the heart but was probably more sensitive to the action of insulin.

The trichloroacetic-acid extractable fraction of the glycogen was more variable physiologically than the non-extractable portion in all the tissues

examined. In the heart, all the variations induced by the experimental conditions employed here appeared to occur exclusively in the extractable portion of the glycogen.

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CONCENTRATION GRADIENTS FOR RADIOIODIDE IN UNBLOCKED THYROID GLANDS OF RATS: EFFECT OF PERCHLORATE[1]

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THE iodide-trapping mechanism of the thyroid provides a concentrated substrate for subsequent organic iodinations within the gland. Obliteration of this function, by anions such as thiocyanate or perchlorate, results in diminished formation of hormone, goiter, and myxedema (Barker, 1936; Astwood, 1943; Rawson, Tannheimer and Peacock, 1944; Wolff *et al.*, 1946; VanderLaan and VanderLaan, 1947; Wyngaarden, Wright, and Ways, 1952).

Under conditions of blockade to organic binding, external counting techniques in man have repeatedly demonstrated within the gland concentrated radioiodide which can readily be discharged by thiocyanate or perchlorate (Stanley and Astwood, 1948; Stanbury and Wyngaarden, 1952). Similar attempts to demonstrate dischargeable thyroidal radioiodine have met with variable succees when organic-binding has been allowed to proceed (Kirkland, 1954; Berson and Yalow, 1955; Ingbar, 1955; VanderLaan, 1955). Several investigators, having been unable consistently to demonstrate dischargeable radioiodine under the latter conditions, have concluded that organic-binding of concentrated iodide is virtually instantaneous (Berson and Yalow, 1955; VanderLaan, 1955). Thus the gland might be actively concentrating inorganic iodine, but at any moment in time, essentially no exchangeable radioiodide would be available for discharge. The iodide-concentrating mechanism would thus be the sole rate-limiting factor in hormonal synthesis.

In thyroidal tissue of animals and humans in whom organic iodinations have not been blocked, inorganic iodine, both stable and radioactive, has been demonstrated by a variety of analytic techniques (Gutman *et al.*,

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1932; Taurog, Tong and Chaikoff, 1951; Braasch *et al.*, 1955). These quantities of iodide have been sufficient to indicate the apparent existence of thyroid/plasma concentration gradients of variable magnitude (Lein, 1943; Taurog, Tong and Chaikoff, 1951; Wollman and Scow, 1953).

Advocates of the hypothesis of instantaneous binding of iodide have suggested that this inorganic iodine may represent a methodological artefact, *i. e.*, either breakdown of organic to inorganic iodine during fractionation of these moieties, or failure to achieve their complete separation (Berson and Yalow, 1955). This hypothesis cannot explain, however, the observation that 10 minutes following the injection of I^{131} in rats, the specific activity of thyroidal iodide was higher than that of any other iodine fraction (Taurog, Tong and Chaikoff, 1950). Alternatively it has been proposed that this inorganic iodine may exist in a form or locus which differs from that of the iodide utilized in the formation of hormone and discharged by thiocyanate (Berson and Yalow, 1955).

Difficulties inherent in demonstrating, by external counting techniques, discharge of concentrated radioiodide from unblocked glands are numerous, and have been discussed in detail elsewhere (Ingbar, 1955). The present attempts to elucidate this problem have therefore employed direct radioactive analysis of excised thyroid tissue. Animal tissue has perforce been used. By means of a technique hitherto undescribed, perchlorate-dischargeable radioiodide, in quantities sufficient to establish thyroid/plasma concentration gradients, has been demonstrated in the unblocked thyroid glands of rats.

METHODS

General considerations. Two criteria must be met by any procedure employed in investigating iodide-concentrating by thyroid glands in which organic iodinations are proceeding. First, physiological alterations such as anoxia, change in pH, devitalization, or substrate depletion, which might of themselves inhibit organic binding, should be excluded. Therefore, surviving tissue slices could not be employed, and studies were performed in normal animals, undisturbed save for the injection of I^{131} and either normal saline or a solution of sodium perchlorate.

Secondly, the technique employed in fractionating thyroidal radioiodine should completely separate organic from inorganic moieties. In addition, degradation of organically-bound to inorganic iodine during the fractionation procedure should be avoided.

Method. Male rats of the Sprague-Dawley strain were employed. Rats were injected intraperitoneally at 5 minute intervals with 10 μc. of carrier-free inorganic I^{131}. They were then divided into two groups, one to receive 0.1 m. mol. of sodium perchlorate intraperitoneally, the other to receive saline and serve as controls. In Experiment I animals weighed 130–150 gm. and had been maintained on a diet of Purina Laboratory Chow. All animals were killed 80 minutes following injection of I^{131}, experimental rats having received sodium perchlorate and control rats saline 20 minutes earlier. In Experiment II animals weighed 100–110 gm. and had been maintained for 1 week on a low iodine diet.[3] Control rats were sacrificed one hour following injection of I^{131}. Experi-

[3] Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

mental rats were given perchlorate at this time and were sacrificed 20 minutes later. Animals were sacrificed *ad seriatim* according to the following procedure.

Each animal was quickly and lightly anesthetized with ether. The abdomen was opened and 2 ml. of blood were withdrawn from the abdominal aorta into heparinized syringes. The thyroid gland was exposed, excised, and quickly weighed on a torsion balance. It was then placed inside a dialysis sac containing 2 ml. of ice-cold human serum. Thereafter thyroid glands and surrounding solutions were continuously refrigerated. Dialysis sacs were closed and suspended in 10 ml. of ice-cold physiological saline solution contained within 4-dram glass vials. These were sealed with paraffin-coated rubber stoppers and were placed on a continuously-rocking platform in the refrigerator. Following 48 hours of dialysis, radioactivity of the entire vial was assayed. Two equal aliquots of each dialysate were then withdrawn. The radioactivity of the first was measured. Following acidification of the second aliquot, NaI and KIO_3 were added, and the iodine evolved was extracted with chloroform. Radioactivity of the chloroform extract was compared with that of the first aliquot of dialysate. Radioactivity was assayed in diluted specimens of the plasma obtained from rats' aortic blood. All counts were obtained in a well-type scintillation counter (sodium-iodide crystal-thallium activated) and were related to a suitable dilution of the administered dose of I^{131}. Sufficient counts were obtained to reduce the probable counting error to a maximum of 1%.

Concentration Gradients (C. G.) for inorganic iodide were calculated according to the following formula:

$$\text{C.G.} = \frac{(\text{dialyzable } I^{131} \text{ within bag}) + (I^{131} \text{ in dialysate})^{4}}{I^{131}/\text{ml. plasma} \times \text{thyroid wt. (gm.)}}$$

Control experiments (v.i.) revealed that iodide apparently binds to protein within the sac and is not completely dialyzable. In eight experiments in which inorganic I^{131} was added either to the inside or the outside of the dialysis sac, the ratio I^{131}/ml. plasma/I^{131}/ml. dialysate averaged 1.20. Furthermore, following dialysis the volume of the contents of the bag had increased to 2.2 ml. and of the dialysate had decreased to 9.8. Therefore,

$$\text{dialyzable } I^{131} \text{ within bag} = 2.2 \times 1.20 \times I^{131}/\text{ml. dialysate}$$
$$I^{131} \text{ in dialysate} = 9.8 \times I^{131}/\text{ml. dialysate}$$

$$\text{C.G.} = \frac{12.44 \times I^{131}/\text{ml. dialysate}}{I^{131}/\text{ml. plasma} \times \text{thyroid (gm.)}}.$$

Thyroidal content of organically-bound radioiodine was calculated according to the following formula:

$$\text{organic } I^{131} \text{ (\% dose)} = \text{total } I^{131} \text{ in vial (\% dose)} - 12.44 \times I^{131}/\text{ml. dialysate (\% dose)}$$

Statistical methods employed were those described by Snedecor (1946).

Validation of Method. By using living animals, alterations in the physiological or biochemical status of thyroidal tissue, *i.e.*, anoxia, changes in pH, depletion of substrates, such as might occur during accumulation of I^{131} in tissue slices, have been avoided. Thus at least until induction of ether anesthesia, there should have been no reason for organic-binding to have been blocked. Total lapse of time between induction of anesthesia and excision of the thyroid gland did not exceed 3 minutes, and not more than 1 minute elapsed between completion of arterial puncture and removal of the thyroid gland. In

[4] It should be emphasized that the term, "I^{131}/ml. plasma," which appears in the denominator of this expression, refers to the radioiodine content of the rats' plasma, obtained at the time of sacrifice, and not to the radioactivity of the human serum within the dialysis sac.

TABLE 1. INFLUENCE OF ADDED DIIODOTYROSINE AND THIOSULFATE ON THYROID/PLASMA CONCENTRATION GRADIENTS FOR I^{131}, AS DETERMINED IN THE DIALYSIS SYSTEM*

Number of animals	Compound added	T/P ratio	% dialysate I^{131} oxidizable
6	—	16.8 ± 4.8	97.2 ± 3.2
6	diiodotyrosine 5 mg.	15.2 ± 4.3	96.8 ± 2.1
6	sodium thiosulfate 25 mg.	13.7 ± 4.5	94.3 ± 5.0

* Values represent mean ± std. dev. for each group.

almost all instances, the blood removed was well oxygenated. Duration of anoxia with the thyroid *in situ* was therefore very brief. However, even if it be assumed that ether anesthesia immediately and completely blocked organic-binding, it is unlikely that sufficient time could have elapsed prior to thyroidectomy to have permitted the establishment of concentration gradients for radioiodide of the magnitude observed (Wollman, 1954).

Immersion of thyroid glands in cold medium, together with progressive anoxia of tissue within the dialysis sac, would be expected to discharge from the gland any iodide concentrated therein, and thereby to bring a halt to further hormonal synthesis (Freinkel and Ingbar, 1955).

Furthermore, low-temperature incubation would be expected to retard the activity of proteolytic (De Robertis, 1941; Alpers, Robbins and Rall, 1955) and deiodinating (Roche *et al.*, 1951) enzymes within the gland.

Radioiodine incorporated into organic moieties within thyroglobulin, even if leached from the gland, should not be dialyzable. Radioiodinated amino acids existing free (Leblond and Gross, 1949; Tong, Taurog and Chaikoff, 1951) within the gland or liberated by proteolysis would be expected to bind, at least in part, to serum proteins, and thus be rendered non-dialyzable. Although the behavior of monoiodotyrosine with regard to protein-binding is not known, thyroxine and diiodotyrosine cannot be dialyzed from serum to which they have been added (Trevorrow, 1939). At any rate, confirmation of the inorganic nature of radioiodine in the dialysate could be obtained by oxidation and chloroform extraction (Morton and Chaikoff, 1943).

Preliminary experiments were designed to evaluate the validity of these assumptions concerning the experimental method (Tables 1 and 2).

Continued formation of hormone during dialysis would deplete glandular radioiodide and would thereby diminish apparent thyroid/plasma gradients. Thyroid glands were therefore removed from 5 rats not given I^{131}. They were then placed in dialysis sacs containing ice-cold human serum plus added inorganic radioiodine. After forty-eight

TABLE 2. THE INFLUENCE OF DIIODOTYROSINE AND THIOSULFATE ON THE DIALYSIS OF INORGANIC I^{131} FROM HUMAN SERUM*

Number of tubes	Compound added	Serum/dialysate I^{131} ratio	% dialysate I^{131} oxidizable
8	—	1.20 ± .05	97.3 ± 2.8
8	diiodotyrosine 5 mg.	1.17 ± .07	98.6 ± 2.0
8	sodium thiosulfate 25 mg.	1.24 ± .07	94.3 ± 4.5

* Values represent mean ± std. dev. for each group.

hours of dialysis, direct counting of blotted thyroid glands revealed virtually no incorporation of I^{131}. Dialysates demonstrated the same radioactivity as control tubes containing no thyroid glands.

Deiodination of mono- or diiodotyrosine, either existing free within the gland or resulting from continued proteolysis of thyroglobulin, would provide dialyzable radioiodide. This possibility was assessed in two ways. First, 5 rats were injected intraperitoneally with 10 μc. of inorganic I^{131}. Twenty-four hours later thyroid glands were excised and subjected to standard experimental procedure. By this time, only negligible quantities of inorganic radioiodine should have been present in the plasma and thyroid gland, even were concentration gradients for iodide being maintained. The data obtained were consistent with this prediction. Although total thyroidal radioactivity was considerably greater in the 24-hour than in the 80-minute group, dialyzable radioactivity in the former group was considerably less. Only $0.2 \pm 0.1\%$ of total thyroidal radioactivity was calculated to be inorganic in the 24-hour group. Of total thyroidal radioactivity in the 80-minute group $4.0 \pm 1.2\%$ was inorganic.

The foregoing observations indicated that deiodination does not contribute materially to the apparent glandular content of dialyzable radioiodine. This was further assessed in the following manner. Concentration gradients for inorganic iodide in thyroid glands of rats killed at 80 minutes were measured according to the standard procedure, and were compared with gradients obtained when serum within the dialysis sac was enriched with 5 mg. of stable diiodotyrosine. Specific radioactivity dilution should diminish the liberation of radioiodide resulting from deiodination of glandular diiodotyrosine. No difference could be discerned in concentration ratios observed in these two groups.

Direct exchange of inorganic radioiodine with stable, organically-bound iodine would be expected to reduce apparent thyroid/plasma concentration gradients. Conversely exchange of stable inorganic iodine for radioactive organically-bound iodine would augment apparent gradients. No alteration in calculated thyroid/plasma gradients was induced by adding to the system diiodotyrosine or sodium thiosulfate. Similarly, neither agent altered the radioactivity which could be dialyzed from human serum to which inorganic I^{131} had been directly added. In all instances, recovery of dialyzed radioiodine was virtually complete following oxidation and chloroform extraction. Exchange reactions were therefore deemed to be negligible in this sytem.

Finally, the dialyzability of inorganic iodide was assessed. When normal saline solutions were placed both inside and outside the dialysis sac, concentrations of added I^{131} were equal on both sides of the membrane. No binding of I^{131} to the dialysis membrane could be demonstrated.

Other experiments were performed in a "control system." Human serum was placed on the inside and saline on the outside of the dialysis sac. Inorganic I^{131} was added directly, and no thyroid glands were introduced. Under these conditions, the concentration of radioactivity within the dialysis sac consistently exceeded that of the dialysate. The ratio plasma/dialysate radioactivity ranged from 1.14 to 1.31, and averaged 1.20 (std. dev., 0.06). In all instances, distribution of radioactivity was similar, regardless of whether radioiodide had been added to the inside or the outside of the dialysis sac. Thus equilibrium dialysis had been achieved. Oxidation and chloroform extraction confirmed the inorganic nature of the dialyzed radioactivity in all instances. However, precipitation of proteins precluded use of this procedure in the case of dialyzed serum. This preponderance of radioactivity in association with serum proteins was not altered by addition of thiosulfate, and therefore did not represent true iodination of proteins. Rather, it was ascribed to the reversible binding of iodide by serum proteins (principally

albumin), similar to that described for other anions (Scatchard, Batchelder and Brown, 1946; Scatchard, Scheinberg and Armstrong, 1950). The average value of the ratio, 1.20, was therefore employed in calculating the quantity of radioiodide contained within the dialysis sac.

The proportion of chloride or thiocyanate bound by serum albumin decreases as the concentration of anion is augmented (Scatchard, Batchelder and Brown, 1946; Scatchard, Scheinberg and Armstrong, 1950). It might be anticipated, therefore, that loss of iodide from the thyroid gland into the dialysis system would reduce the ratio in animals not given perchlorate. However, it may be calculated that the quantity of iodide contained within the gland (Taurog, Tong and Chaikoff, 1951) would contribute relatively little to the iodide already contaminating the saline solution (Morton, Chaikoff and Rosenfeld, 1944). Furthermore the preponderant volume of the dialysate, compared to that of the dialyzed serum, would minimize errors in the calculation of thyroid/plasma I^{131} gradients, introduced by mistaken evaluation of the magnitude of iodide-binding by serum proteins.

RESULTS

In Experiment I, in which rats were maintained on Purina Laboratory Chow, thyroid/plasma gradients for inorganic radioiodine averaged 16.2 ± 6.8 (mean $\pm$ std. dev.) (Table 3). Gradients were significantly

TABLE 3. INFLUENCE OF SODIUM PERCHLORATE ON THYROID/PLASMA GRADIENTS FOR RADIOIODIDE IN RATS NOT GIVEN AGENTS WHICH INHIBIT ORGANIC IODINATIONS*

Group	Number of animals	Inorganic I^{131} (% dose/gland)	Thyroid wt. (mg.)	Plasma I^{131} (% dose/ml.)	T/P ratio	% Dialysate I^{131} oxidizable	Organic I^{131} (% dose/gland)
Experiment 1. Rats receiving standard laboratory ration							
Control	7	$0.177 \pm .083$	14.5 ± 1.5	$0.80 \pm .05$	16.2 ± 6.8	97.4 ± 3.2	4.2 ± 0.7
Perchlorate	8	$0.035 \pm .019$†	15.1 ± 3.4	$0.96 \pm .05$†	3.2 ± 1.3†	94.8 ± 4.8	3.0 ± 0.7‡
Experiment 2. Rats receiving low iodine diet							
Control	5	$0.341 \pm .161$	15.4 ± 3.6	$0.76 \pm .05$	28.3 ± 9.8	98.1 ± 2.6	8.3 ± 1.2
Perchlorate	5	$0.066 \pm .027$‡	13.6 ± 1.3	$1.04 \pm .06$†	4.7 ± 1.6†	95.3 ± 4.8	9.6 ± 1.4

* Values represent mean $\pm$ std. dev. for each group.
† Value significantly different from control ($p < .001$).
‡ Value significantly different from control ($p < .01$).

depressed following the administration of sodium perchlorate (3.2 ± 1.3; $p < .001$). The concentration of I^{131} in plasma was significantly greater and total thyroidal accumulation of radioiodine significantly less in animals given perchlorate than in the control group.

In Experiment II, in which rats were maintained on a low iodine diet for one week, control gradients averaged 28.3 ± 9.8, a value significantly greater than that found in animals receiving standard laboratory ration ($p = .02$) (Table 3). Gradients were again significantly diminished by perchlorate (4.7 ± 1.6, $p < .001$). Total thyroidal accumulation of I^{131} in perchlorate-treated animals was slightly, though not significantly, greater than in controls. However, the concentration of I^{131} in the plasma of the former animals was significantly increased.

Recovery of dialyzed radioiodine following oxidation and chloroform extraction averaged 96.4 per cent in all experiments.

DISCUSSION

The method herein described affords a convenient means of separating thyroidal inorganic from organically-bound iodine. Proteolysis and deiodination, as well as exchange reactions, do not appear to contribute materially to measured iodide.

By means of the present technique, inorganic radioiodine has been demonstrated in unblocked thyroid glands of rats given I^{131} 60 to 80 minutes prior to sacrifice. Concentration gradients of considerable magnitude between thyroidal and plasma radioiodide have been demonstrated. This iodide is not metabolically inert, since it may readily be discharged by perchlorate, and its discharge is associated with reduction of the subsequent thyroidal accumulation of radioiodine.

The findings in Experiment II, in which rats were maintained on low-iodine diet, indicate that the existence of thyroid/plasma gradients for inorganic iodide is not dependent on partial inhibition of organic-binding resulting from a large dietary intake of iodine (Wolff and Chaikoff, 1948).

In Experiment II, perchlorate was injected into experimental rats at the same time as control animals were sacrificed. Twenty additional minutes were allowed to elapse before perchlorate-treated rats were killed. Thyroid glands of the latter group continued to accumulate I^{131}, since their total thyroidal content of I^{131} was slightly greater than that of controls. Continued accumulation of radioiodine following injection of perchlorate may be ascribed to failure of this agent to abolish concentration differentials completely, instantaneously, or both. It is apparent, however, that external counting techniques would not have revealed a diminution in total thyroidal radioactivity, despite loss of inorganic radioiodine from the gland, as demonstrated by the dialysis technique.

It should be noted that radioactivity in the plasma of perchlorate-treated animals was greater than in controls, despite the greater thyroidal accumulation of I^{131} in the former group, and the longer lapse of time between administration of radioiodine and sacrifice. This apparent discrepancy is perhaps explained by return of radioiodine to the circulation from sites, such as the gastrointestinal tract, wherein it had been concentrated (Mason and Bloch, 1950; Honour, Myant and Rowlands, 1952; Freinkel and Ingbar, 1953).

If freely-exchangeable, perchlorate-dischargeable iodide, destined to be utilized in subsequent organic iodinations, is indeed present in unblocked thyroid glands of rats, then organic-binding of thyroidal iodide cannot be instantaneous. This conclusion is consonant with the observations of other workers, which indicate that a large percentage of the inorganic I^{131} which enters the thyroid gland of the rat returns to the plasma without undergoing organification (Nadler and Leblond, 1955).

Findings in rats can be extrapolated to man only with considerable

caution. However, it seems unlikely that organic-binding is any less rapid in the rat than in man. It is apparent, however, that if the rate of organic binding is finite, it is potentially susceptible to variation. Thus the thyroidal organic-binding reactions may share with the iodide-concentrating mechanism a rate-limiting role in hormonal synthesis.

SUMMARY

Low temperature dialysis in human serum has been employed to separate inorganic from organically-bound radioiodine in excised thyroid glands of rats.

By means of this technique, concentration gradients for inorganic I^{131} have been demonstrated in thyroid glands of rats in whom organic iodinations were proceeding unimpeded. Concentration gradients were diminished by injections of sodium perchlorate.

It is concluded that thyroidal organic-binding reactions proceed at a finite rate, and may play a rate-limiting role in hormonal synthesis.

Acknowledgment

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THE EFFECT OF CORTICOSTEROID INJECTION ON ALDOSTERONE SECRETION[1]

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THE isolation of aldosterone from adrenal extracts (Simpson and co-workers, 1953; Mattox and Mason, 1953; Knauff, Nielson and Haines, 1953) and the demonstration of its presence in the adrenal effluent (Simpson, Tait and Bush, 1952; Farrell and co-workers, 1953, 1954) have aroused interest as to its role in normal physiology and the factors which regulate its secretion. Much is known as to its biological activity (Swingle and co-workers, 1954; Gaunt and co-workers, 1954; Gross and Gysel, 1954). The urinary content of the steriod has been the subject of considerable study and has been found to be altered in a variety of pathological conditions (Singer and Venning, 1953; Singer and Wener, 1953; Luetscher and Johnson, 1954; Luetscher, Neher and Wettstein, 1954; Cope and Garcia-Llaurado, 1954; Gordon and co-workers, 1954; Axelrad and co-workers, 1955; Neher and Wettstein, 1955). Although these studies suggest a role for aldosterone in disease states, the definitive physiological mechanism which regulates the rate of secretion of this important steroid has remained obscure.

It has been demonstrated that there is little reduction in aldosterone secretion after hypophysectomy (Farrell, Rauschkolb and Royce, 1955; Farrell, Rauschkolb and Koletsky, 1955; Rauschkolb and co-workers, 1955). In support of this finding is the evidence of Luetscher and Axelrad (1954) that aldosterone occurs in the urine of patients with panhypopituitarism, as well as the earlier experiments of Swann (1940), Deane and Greep (1946) and Lane and de Bodo (1952) relative to the maintenance of nearly normal electrolyte metabolism in hypophysectomized animals.

Ingle (1938), Sayers and Sayers (1949) and Wilkins and collaborators (1951) demonstrated that the administration of corticosteroids inhibits the release of pituitary ACTH and in turn the secretion of steroids by the adrenal. The administration of cortisone has also been shown to markedly depress the synthesis of pituitary ACTH as evidenced by almost complete absence of ACTH in the gland after long-term cortisone administration in

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the dog (Farrell and Laqueur, 1955). The administration of large amounts of corticosteroids appears to be the physiological equivalent of hypophysectomy. It was therefore of interest to determine the effect of prolonged steroid administration on the rate of secretion of aldosterone by the adrenal. The present study demonstrates that while treatment with cortisone, followed by 17-hydroxycorticosterone over a period of 5 weeks causes profound suppression of secretion of 17-hydroxycorticosterone, corticosterone and 11-desoxy-17-hydroxycorticosterone, it has no significant effect on the rate of secretion of aldosterone.

METHODS

Seven large male dogs were employed. Four dogs were given daily subcutaneous injections of steroid. Cortisone, 100 mg. per day was injected for 8 days, followed by 17-hydroxycorticosterone, 100 mg. per day for 28 days. The remaining three dogs received the injection vehicle and served as controls. At the end of the 5-week experimental period the dogs were anesthetized with sodium pentobarbital and bled from the adrenal vein. Heparin was administered intravenously to prevent clotting. Bleeding was carried out over a 4-hour period, at the end of which time the animals were sacrificed. The pituitaries were removed for assay of ACTH content and the adrenals preserved for histological examination.

The adrenal venous blood, collected at 0° C., was promptly extracted with chloroform and aldosterone, 17-hydroxycorticosterone, corticosterone and 11-desoxy-17-hydroxycorticosterone were isolated by paper chromatographic methods described earlier (Farrell, Rauschkolb, Royce and Hirschmann, 1954). Aldosterone was isolated in the diacetate form. Evidence that the isolated substance was aldosterone diacetate is as follows: An aliquot of each sample from the control and steroid-treated animals chromatographed as a single band when mixed with authentic aldosterone diacetate. The sulfuric acid chromogen was identical with that of the authentic compound. Aliquots of the aldosterone fractions from the control animals and the steroid-treated animals were hydrolyzed (acetylcholinesterase in glycylglycine buffer 30°, 4 hours) and the hydrolysis mixtures were bioassayed in adrenalectomized rats for sodium-retaining activity. The characteristic high potency of aldosterone was found. The ratio of potency of the aldosterone fractions from cortisone-treated animals as compared to that from controls was 3.75 ± 2.5, indicating that the steroid isolated from the treated dogs was as potent as that isolated from the controls.

Assay for ACTH content of the pituitary glands was by the method of Sayers and co-workers (1948). The pituitary glands were ground in the frozen state in 1/10 N HCl in saline and diluted to appropriate doses with 1/100 N HCl for injection.

RESULTS AND DISCUSSION

The adrenals of the treated dogs differed markedly from the controls. The organs were considerably reduced in size. The average adrenal weight (left plus right) of the control dogs was 3.0 ± 0.45 gm., while that of the steroid-treated dogs was 1.25 ± 0.1. The reduction in adrenal weight was statistically significant, $p < 0.01$. The reduction in size was due to cortical atrophy involving both the fascicular and reticular layers. In the fasciculata the cells were smaller, the nuclei more crowded and pyknotic than normal; the cytoplasm was contracted and less vacuolated. There was loss

of the columnar grouping of the cells. Similar atrophic changes were present in the reticular layer. The glomerulosa was well preserved and not significantly altered in comparison with the control adrenals. In fact, this layer appeared thicker than that of the control organs. This observation would correlate with the finding of continued production of aldosterone by the adrenal after prolonged steroid therapy. It also indicates that the integrity of the glomerulosa cells is not dependent on ACTH production. No significant change was observed in the medulla.

The pituitary content of ACTH was markedly reduced. The ratio of potency, treated/control, was $0.19 \pm .13$, a reduction, on the average, of approximately 80%. The finding of a marked reduction in ACTH content of the pituitaries of the steroid-treated animals is in accord with an earlier report from this laboratory.

The results of analyses for aldosterone, 17-hydroxycorticosterone, corticosterone and 11-desoxy-17-hydroxycorticosterone are shown in Table 1. Except for aldosterone, the rates of secretion of these steroids were

TABLE 1. SECRETION OF STEROIDS (μG./KG. BODY WEIGHT/HOUR) BY THE ADRENAL FOLLOWING CORTICOSTEROID ADMINISTRATION

Steroid	Control dogs				Steroid treated dogs				
	1	2	3	Mean	1	2	3	4	Mean
17-Hydroxycorticosterone	22.2	16.5	19.3	19.33	.40	.89	.46	.19	.48
Corticosterone	4.08	7.94	3.60	5.21	2.2	.38	1.48	1.25	1.33
11-Desoxy-17-hydroxycorticosterone	4.96	6.16	6.82	5.98	.77	.25	.71	.60	.58
Aldosterone	.211	.217	.250	.226	.293	.295	.450	.107	.286

profoundly reduced and were of the order of magnitude found after complete hypophysectomy (Rauschkolb, Farrell and Koletsky, 1955). These findings are consistent with the view that prolonged steroid administration induces almost complete cessation of pituitary production of ACTH.

The secretion of aldosterone was slightly higher, on the average, in the steroid-treated animals than in the controls. However, because of variation in individual rates this apparent increase is not statistically significant. That steroid treatment did not diminish the rate of aldosterone secretion, as contrasted to its effects on the other steroids, is strikingly apparent.

The results of this study are in agreement with earlier reports that aldosterone secretion is not dependent on ACTH. It seems apparent that both increases and decreases in the rate of production of this physiologically important steroid may occur independently of the other adrenal steroids.

Therapy with cortisone or 17-hydroxycorticosterone would not of itself be expected to alter aldosterone output. The decrease in urinary excretion of aldosterone of nephrotic children during or following cortisone treatment (Luetscher and Deming, 1950) does not seem, in the light of our findings, to be due to the pituitary-suppressing action of the injected steroid. As suggested by Luetscher (1954), the effects may very well be related to the anti-inflammatory action of the hormone.

It is worthy of note that the secretion of aldosterone following steroid treatment is well within the normal range, whereas the rate following surgical hypophysectomy, although at physiologically important levels, appears to be somewhat diminished. After acute (6–10 hours) hypophysectomy the rate of adrenal production of aldosterone is about $\frac{1}{2}$ of normal. The level, 6 days after hypophysectomy, is about $\frac{2}{3}$ of normal. Two possibilities may be considered to explain this finding: (1) Complete hypophysectomy, with the removal of all pituitary factors, may lead to a diminution of aldosterone synthesis secondary to a generalized reduction in tissue metabolic activity, or (2) surgical removal of the pituitary may result in damage to a nearby central nervous system structure which acts as the primary regulator of aldosterone secretion. Evidence in favor of either possibility is inadequate to permit a definitive statement at this time.

SUMMARY AND CONCLUSIONS

Administration of cortisone and 17-hydroxycorticosterone, 100 mg. per day, over a period of 5 weeks does not reduce the rate of aldosterone secretion by the adrenal of the dog, as determined by isolation of this steroid from adrenal venous blood. The rates of secretion of 17-hydroxycorticosterone, corticosterone and 11-desoxy-17-hydroxycorticosterone are markedly reduced, and are of the same order of magnitude as after complete hypophysectomy. The pituitary content of ACTH is markedly diminished. The corticosteroid treatment results in a marked cortical atrophy of the adrenals which does not involve the glomerulosa layer.

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EFFECT OF CASEIN ON IODIDE METABOLISM[1]

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IN 1950 we reported (Van Middlesworth, 1950) that we had raised rats on purified casein diets containing very little iodide (20–50 μg. iodide/kg. of diet, Van Middlesworth, 1951) and no goiter resulted. Since then we have attempted to elucidate the mechanism of this phenomenon (Van Middlesworth, 1953). Axelrad and Leblond (1954) recently reported that casein prevented goiter in mice fed a low iodide diet.

MATERIALS AND METHODS

To study the mechanism of the casein effect on iodide metabolism, Long-Evans rats weaned to their particular diet were fed a goiter producing commercial cereal diet[2] of initially fixed I^{131} specific activity (0.02 or 0.05 μc. I^{131}/gm. of diet) as described previously (Van Middlesworth 1952; Van Middlesworth and Intoccia, 1954; Van Middlesworth 1955). These rats were maintained in metabolism cages and their excreta were collected each day and analyzed for I^{131}. After these animals reached iodine equilibrium the casein content of the diet was changed. Distilled water was permitted *ad libitum* and the animal room was maintained at 70–74° F.

The casein diet[3] and commercial cereal[1] were repeatedly analyzed for iodide (Chaney, 1940; Barker *et al.*, 1951; Zak *et al.*, 1952). For both diets the total iodide content was close to the limit of sensitivity of the analytical methods (0.015 μg. per gm.). The small iodide content of the reprecipitated casein was unchanged by prolonged extraction (Van Middlesworth *et al.*, 1953) which may indicate that the iodide was bound to the casein.

The possibility that casein was slightly iodinated while being synthesized by the mammary gland was investigated. Lactating mongrel dogs were injected with 10 mc. I^{131} and milk was expressed 1–4 hours later. The plasma and milk of the dogs were dialyzed and studied by paper electrophoresis in veronal buffer at pH 8.5. The electrophoretic strips were studied by counting and by radioautography. The protein fractions were extracted by acid-butyl alcohol and the extracts analyzed by paper chromatography using the butyl alcohol solvents of Gross *et al.*, 1950.

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[1] Presented before American Goiter Association, Oklahoma City, Oklahoma, April 28–30, 1955.

[2] Produced by Mead Johnson and Company, Evansville, Indiana and marketed under the trade name of Pablum Mixed Cereal. This cereal is stated to contain "Wheat meal (farina), oatmeal, yellow corn, wheat germ, tribasic calcium phosphate, powdered alfalfa leaf, dried yeast, sodium chloride, thiamine hydrochloride, riboflavin, and reduced iron." The mixture is "precooked" and dried to a moisture content of 7%. This cereal was purchased on the open market, one month's supply at a time from July 1953 through January 1955.

[3] Composition: Vitamin-free casein (0.11 μg. iodide per gm., purchased from Nutritional Biochemicals Corp., Cleveland, Ohio) 13% or 26%; sucrose (Domino) 68% or 55%; vegetable fat (Crisco) 10%; purified salts and crystalline vitamins.

Van Middlesworth has shown recently (1955) that the casein-sucrose low-iodide diet did not produce goiter but the commercial cereal diet resulted in thyroids which average 7 times normal size. The addition of casein to the commercial cereal diet prevented goiter even though the mixture was still very low in iodide content (calculated maximum was 0.04 to 0.05 μg. total iodide/gm. of diet).

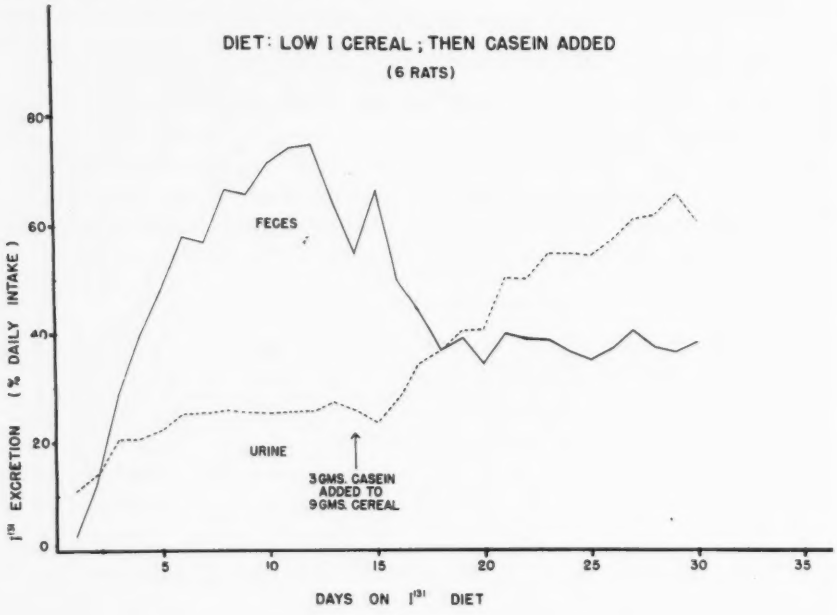
DIET: LOW I CEREAL ; THEN CASEIN ADDED
(6 RATS)
FECES
URINE
3 GMS. CASEIN ADDED TO 9 GMS. CEREAL
I131 EXCRETION (% DAILY INTAKE)
DAYS ON I131 DIET

FIG. 1

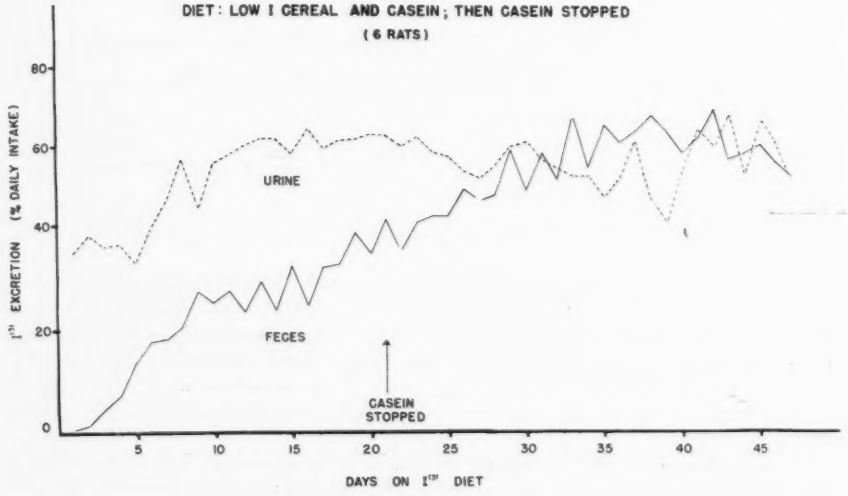

FIG. 2

RESULTS

Figure 1 shows the iodide metabolism in rats fed the I^{131}-tagged commercial cereal diet until I^{131} equilibrium was attained. Approximately 70% of the daily iodide intake was excreted in the daily feces. The fecal iodide was interpreted to indicate dietary iodide which had passed through the thyroid gland and become incorporated into organic compounds (Van Middlesworth *et al.*, 1955). Therefore, as Figure 1 shows, most of the daily dietary iodide was utilized by the thyroid until casein was added to the cereal diet. The added casein appeared to reduce thyroid function, according to this interpretation, since the relative urinary excretion of iodide was increased 350% and the fecal excretion was sharply decreased. After the casein was added, the sum of daily urine and fecal I^{131} was less than the daily intake; this indicated a positive iodide balance. The balance was calculated for each day and these values were added progressively. The positive balance from casein is shown in Figure 3.

The radioactivity of rats in Figure 1 had decayed to very low levels after

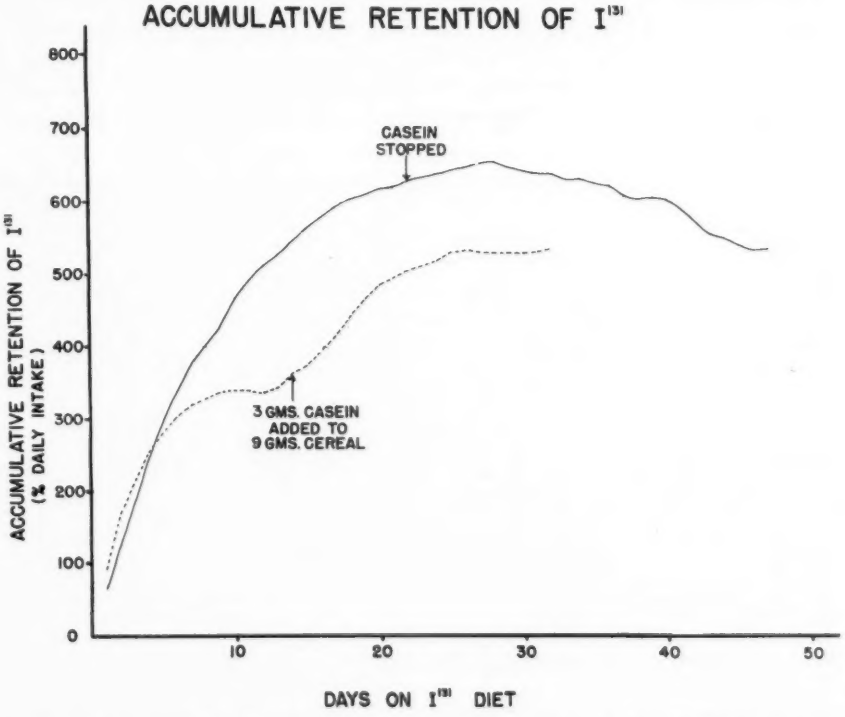

FIG. 3. Accumulative retention of I^{131} in rats shown in Figures 1 and 2. (Daily retention was determined by adding the daily urine and fecal I^{131}, expressed as percentage of the daily dose, and subtracting the sum from 100%. These daily retentions were progressively added to give the accumulative retention. (Endocrinology, in press.)

30 days. Therefore, to study casein withdrawal a second group of animals had been maintained similarly to those in Figure 1. Casein was withdrawn from the diet of this second group (Fig. 2) after I^{131} equilibrium. A longer time was required for the excretion patterns of these animals to approach an initial plateau but after 20 days their urinary and fecal I^{131} were similar to the terminal levels in Figure 1. After casein was discontinued the urinary excretion changed only slightly and the fecal excretion increased very slowly. After 25 additional days the excretion pattern was still quite different from the initial equilibrium level of Figure 1. These data suggest that the effects of casein on iodide metabolism were prolonged after removal of casein from the diet. Figure 3 shows a slow, progressive negative iodide balance (Van Middlesworth, 1955) after discontinuing the casein supplement.

It was considered unlikely that inorganic iodide in the purified casein could account for the alterations in iodide metabolism shown to be due to casein. The iodide in purified casein appeared to be bound to the protein (Van Middlesworth *et al.*, 1953). This suggests the possibility that the digestion of casein may result in a compound which effectively inhibits thyroid hypertrophy.

To investigate casein-bound iodide, milk from I^{131} injected dogs was studied by paper electrophoresis (Fig. 4). All the protein fractions contained bound I^{131}.

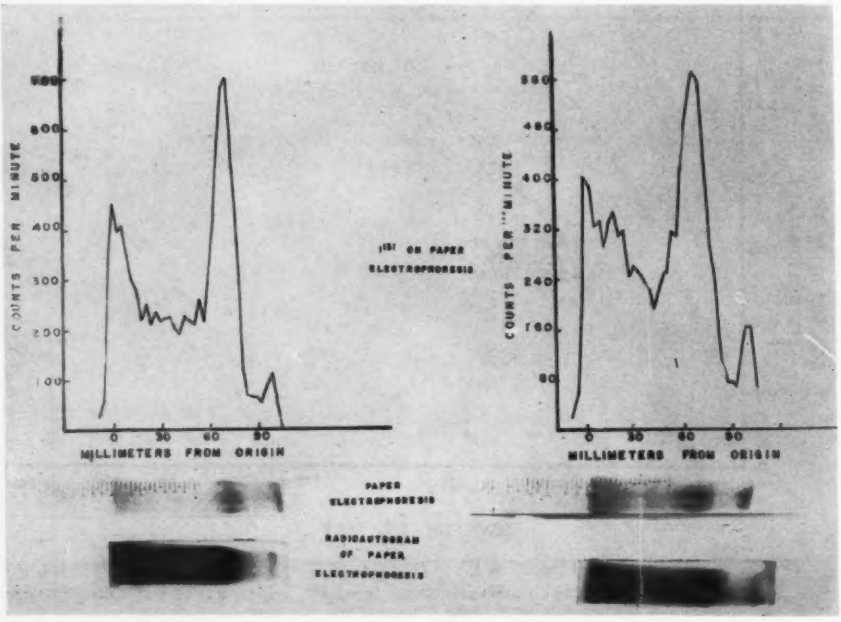

FIG. 4. Paper electrophoresis of dialyzed radioactive milk from dog injected with I^{131} four hours before milk sample collected.

DISCUSSION

Purified casein added to a goiter producing diet prevented goiter and produced easily demonstrated alterations in iodide metabolism. The reduction in fecal iodide due to casein has been interpreted as indicating reduced thyroid release of organic iodinated compounds. The resulting positive iodide balance from casein and the prolonged casein effect after discontinuing casein suggests that casein depressed the thyroid iodide trapping mechanism less than it depressed the release of iodinated compounds. An antigoitrogenic effect of casein has been reported by Remington (1937) and by Axelrad and Leblond (1954). Remington found large amounts of iodide in his casein and stated that the goiter prevention was probably due to this iodide. Purified casein contained no demonstrable inorganic iodide and very little iodide could be released even with complete incineration of the protein. It is suggested that the digestion of casein may release a substance which inhibits thyroid hypertrophy.

SUMMARY

Dietary purified casein has been shown to be an effective goiter preventative agent. No inorganic iodide was found in the casein but a very small amount of organic iodide was demonstrated.

Milk proteins were shown to be iodinated in the synthesis of milk. It is proposed that the casein-iodine combination, when digested, results in a substance which can prevent thyroid hypertrophy.

Acknowledgments

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STUDIES ON THE BLOOD GLUCOSE AND PANCREATIC ISLETS OF LIZARDS[1]

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REMARKABLY little is known about normal or experimental blood glucose levels or the behavior of the pancreatic islet cells in reptiles. The present study concerns the determination of the blood glucose levels and the histological aspects of the pancreatic islet cells in several species of lizards in the fed and fasted states, and after alloxan and insulin administration.

MATERIALS AND METHODS

Freshly captured specimens of the following species of lizards were used in this study: *Anolis carolinensis, Callisaurus draconoides, Cnemidophorus tigris, Eumeces fasciatus, E. obsoletus, E. skiltonianus, Gerrhonotus multicarinatus, Sceloporus magister, S. occidentalis, Uta stansburiana,* and *Xantusia vigilis.*

Blood samples were obtained from the carotids after decapitation and the blood glucose determined by averaging the values of at least three specimens from each animal. True blood glucose values were determined by the Somogyi copper-iodometric method (Somogyi, 1945).

The pancreases were excised and fixed in Bouin's, Helly's, Dawson's (formol-sublimate), formalin, and acetic osmic bichromate solutions. After paraffin embedding, 4μ sections were stained with Gomori's (1941) chromalum hematoxylin and phloxine, Gomori's (1950) aldehyde fuchsin, hematoxylin and eosin, and Heidenhain's azan methods. For routine purposes the most satisfactory results were obtained with Bouin's fixative followed by Gomori's chromalum hematoxylin and phloxine staining.

Fasting blood sugars were determined on animals 7 to 28 days after the last meal. In all cases the intestines were devoid of any food material.

It was determined that 3 to 5 times more alloxan is required in lizards than in mammals to produce islet cell changes. Hence, 300 mg./kg. of alloxan was injected intraperitoneally every other day for 4 or 5 doses. Animals were sacrificed at 8 and at 14 days after treatment.

An effective shocking dose of insulin (crystalline zinc insulin given intraperitoneally) in most lizards is 2000 units per kg. Animals were given dosages of 1000 to 10,500 units/kg. of insulin and sacrificed after 10, 20, 30, 45, 60, 90, 120, and 300 minutes. Since histological effects of insulin were not apparent after single dosages, one group of 8 animals was treated daily with 2000 units/kg. for 4 days and then sacrificed 3 hours after the last injection.

Blood glucose levels could be determined satisfactorily only on larger lizards (5 to 50 gm.), but the pancreases of all animals were studied histologically.

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TABLE 1. BLOOD GLUCOSE LEVELS IN FASTING LIZARDS

Species	True blood glucose in mg. %
Anolis carolinensis	74.0
Eumeces obsoletus	84.2
Eumeces skiltonianus	90.0
Eumeces obsoletus	91.4
Eumeces obsoletus	93.2
Eumeces obsoletus	94.3
Eumeces obsoletus	99.5
Eumeces obsoletus	110.4
Eumeces obsoletus	113.9

OBSERVATIONS

Observations on blood glucose variations. The fasting blood glucose in various species of lizards varied from 74.0 to 113.9 mg.% (Table 1). These values were determined on animals that had been fasted from 7 to 28 days. The length of the fast within this time did not produce any statistically significant alteration in blood sugar, although a tendency was observed for an early fasting hypoglycemia to occur.

During the week after the last meal the blood sugar level is highly variable, but is well above the fasting level. Postprandial blood glucose values varied from 140.5 to 224.1 mg.% (Table 2). No species or sex differences in blood glucose values were observed on either fasting or fed lizards. The postprandial hyperglycemia present in reptiles indicates that there is considerably less rigid regulation of blood glucose levels in reptiles than in mammals.

Reptiles, like birds, are relatively insulin resistant. Trials in several species of lizards showed that minimal signs of insulin shock occur with dosages of approximately 1000 units/kg. With 2000 to 3000 units/kg. more marked shock symptoms are present. Usually within one minute after intraperitoneal injection of a shocking dose of insulin, lizards show generalized trembling, loss of muscular coordination, mild muscular spasticity, and an increased respiratory rate. These signs may last from 20 minutes to several hours. With larger dosages of insulin (up to 10,500 units/kg.) the

TABLE 2. BLOOD GLUCOSE LEVELS IN FED LIZARDS

Species	True blood glucose in mg. %
Eumeces fasciatus	140.5
Sceloporus occidentalis	142.5
Eumeces fasciatus	143.1
Anolis carolinensis	148.8
Anolis carolinensis	181.6
Anolis carolinensis	184.5
Anolis carolinensis	190.6
Anolis carolinensis	212.8
Eumeces skiltonianus	215.3
Eumeces skiltonianus	224.1

TABLE 3. EFFECT OF INSULIN ON BLOOD GLUCOSE LEVELS IN LIZARDS

Species	Units insulin per kg.	Time in minutes after injection	True blood glucose in mg. %
Eumeces obsoletus	3,500	10	34.0
Eumeces skiltonianus	3,000	10	39.9
Anolis carolinensis	3,000	20	28.0
Eumeces obsoletus	3,000	20	33.6
Eumeces obsoletus	3,000	30	80.4
Anolis carolinensis	3,000	30	84.4
Anolis carolinensis	3,000	45	99.0
Eumeces obsoletus	3,000	60	113.6
Eumeces obsoletus	3,000	60	100.8
Eumeces obsoletus	3,000	60	113.1
Eumeces obsoletus	3,000	90	142.1
Eumeces obsoletus	10,500	120	129.9
Sceloporus occidentalis	3,500	300	135.0
Sceloporus occidentalis	3,500	300	176.5

condition of "shock" is of longer duration, but as yet no animal has become convulsive, comatose, or has expired from insulin overdosage.

Insulin hypoglycemia is apparently of short duration (up to 20 minutes after injection), as animals sacrificed from 30 to 60 minutes after insulin treatment showed normal fasting blood sugar levels. One and one-half to two hours after insulin injection there was a mild hyperglycemia (Table 3). The shock manifestations (muscular incoordination and trembling) are not necessarily related to hypoglycemia as animals still in shock after 2 hours may be hyperglycemic.

Experiments with alloxan dosages indicate that if 600 mg./kg. were given in a single injection, the animals usually died within 24 to 48 hours. If a single dose of 300 mg./kg. was given, no blood sugar or histological effects were observed. With three to five injections of 300 mg./kg. of alloxan given at 48 hour intervals, both blood glucose and islet cell changes were produced. Eight days after alloxan administration (1.2 gm. given in 4 divided doses at 48 hour intervals) only one of five animals was hyperglycemic. Fourteen days after 1.5 gm. given in 5 divided doses, all of 6 animals were hyperglycemic and showed beta cell destruction in the islets (Table 4).

TABLE 4. EFFECT OF ALLOXAN ON BLOOD GLUCOSE LEVELS IN LIZARDS

Species	Alloxan dosage	True blood glucose in mg. %
Eumeces obsoletus	(Total of 1.2 gm./kg.) 300 mg./kg. were given on the	100.9
Eumeces obsoletus	1st, 3rd, 5th, and 7th days of treatment. Sacrificed on	70.3
Eumeces obsoletus	8th day	105.8
Eumeces obsoletus		118.3
Eumeces obsoletus		164.1
Eumeces obsoletus	(Total of 1.5 gm./kg.) 300 mg./kg. were given on the	150.1
Eumeces obsoletus	1st, 3rd, 5th, 7th, and 9th days of treatment. Sacrificed	162.7
Eumeces obsoletus	on 14th day	152.6
Eumeces obsoletus		145.5
Eumeces obsoletus		189.0
Eumeces fasciatus		209.3

Histological observations. As has been previously described by Barrington (1953) and Thomas (1942) in snakes, and by Laguesse (1901, 1902) in lizards, islet tissue is particularly abundant in the splenic lobe of the pancreas. The islets are large, somewhat irregular and variable in shape, and are composed of at least two well differentiated cell types. The alpha cell of the pancreatic islet has fine water soluble granules which stain pink with eosin, ruby red with azocarmine, red with phloxine, and are argyrophilic. The beta cell has very fine alcohol soluble granules which stain light pink with azocarmine, bluish-purple with aldehyde fuchsin, and bluish-black with chromalum hematoxylin. In all species of lizards studied the alpha cells are more abundant than the beta cells. Both cell types are periodic acid Schiff negative. Small faintly basophilic degranulated appearing cells were occasionally encountered. These may be the so-called delta cells. We are of the opinion that transitional stages between the alpha and beta cells do not exist, but that the alpha and beta cells are independent and that degranulated and granulated phases of each cell type may be found.

In normal fed lizards, both the alpha and beta cells show partly degranulated to fully granulated stages. After prolonged starvation (3 to 6 weeks) the alpha cells tend to become more degranulated and the beta cells to be less abundant. Up to two hours after a single shocking dose of insulin, no definite histological changes were evident. However, in animals given a shocking dose every day for four consecutive days we observed degranulation of beta cells and an increase in the granulation of the alpha cells.

Five hours after the oral administration of glucose (3 animals given 7 gm. /kg. as a 5% solution) all animals were hyperglycemic, with blood glucose levels around 200 mg.%, and showed beta cell degranulation.

During the first week of alloxan treatment degranulatory changes were seen in the beta cells. From the 8th to the 14th days after the institution of treatment there was progressive destruction of beta cells and granular storage in the alpha cells coincident with the development of hyperglycemia.

DISCUSSION

So far as we can find, very little is known concerning blood glucose values and histological changes in the pancreatic islet cells in reptiles under different physiological conditions. What little is known in the lower vertebrates is well covered by Bargmann (1939) in Möllendorff's Handbuch.

Preliminary studies on the salamander, *Taricha torosa*, (Wurster, unpublished) indicate that these animals have relatively low fasting blood sugar values (approximately 30 mg.%), and are very sensitive to insulin. The islets of *Taricha* apparently consist largely of beta cells, as few alpha cells are to be seen. Similar low fasting blood glucose levels have been observed in frogs by Smith (1950).

Lizards, on the other hand, have a greater proportion of alpha cells, have higher fasting blood sugar levels, and are remarkably insulin resistant.

Rhaney's (1948) values of 91 ± 4 mg.% for the fasting blood sugar of the king snake, *Lampropeltis*, are similar to the values obtained by us in lizards. Insulin resistance in reptiles has also been observed by Prado (1947) and Houssay *et al.* (1923).

Because of the contrast of low alpha cell content and low insulin susceptibility in amphibians with a high alpha cell content and high insulin resistance in reptiles, it is attractive to postulate that insulin resistance in reptiles (and probably also in birds) may be due at least in part to some factor produced by the alpha cells. Such a substance might be similar to the proposed hyperglycemic factor which may be produced by the alpha cells of mammalian islets. However, the fact that mammals have abundant alpha cells seemingly homologous to those of the reptile and yet are insulin sensitive, would indicate that factors other than alpha cell hormone are involved in insulin resistance or sensitivity.

The fasting blood glucose levels of birds (Sturkie, 1954) are approximately twice that of lizards and snakes, but the degree of insulin resistance is similar. Further studies will probably show that reptiles are the most insulin resistant of all vertebrates, while birds have the highest fasting blood glucose level.

Like birds (Scott *et al.*, 1954), lizards are relatively resistant to alloxan. With sufficient alloxan, however, lizards will develop hyperglycemia in one to two weeks. Garcia Ramos (1944) produced hyperglycemia in turtles with alloxan.

Since the alpha and beta cells of the reptilian pancreatic islets stain and behave similarly to the corresponding cells in the mammalian islets, we believe these cells to be homologous in the two groups. We are in agreement with the conclusions of Thomas (1942) that the alpha and beta cells of reptiles are distinct and separate cells. Because of the apparently opposing physiological roles played by the alpha and beta cells of the pancreatic islets we do not support the contention of Barrington (1953) that these two cell types are but different secretory phases of a single type of cell.

The islet cells of fishes, amphibians, and birds have not been sufficiently studied so that they may be compared with those of the reptile and the mammal.

The histological alterations found in the islet cells of the lizard under different physiological conditions indicate that the beta cell is concerned with insulin production and the alpha cell with a hyperglycemic or a fat metabolizing factor.

That the beta cells are concerned with the production of a hypoglycemic factor (insulin) is indicated by degranulation of these cells after glucose administration, partial atrophy after continuous insulin administration, and hyperglycemia subsequent to beta cell destruction by alloxan.

That the alpha cells of the reptilian islets are concerned with a hyperglycemic or fat metabolizing factor is indicated by degranulation during starvation (when the blood glucose is falling and lipotome fat is being

mobilized and utilized), and by granular storage during the hyperglycemic phase of alloxanization. The observations that alpha cells are in a storage state during chronic insulin administration might appear anomalous. However, since lizards become hyperglycemic within an hour after insulin injection, it may be that alpha cell storage is correlated with a state of compensatory hyperglycemia.

Since reptiles in both their embryonic and adult lives must mobilize and use large quantities of stored fat, it seems likely that the pancreatic alpha cell which is already active in the 4 mm. lizard embryo (Miller, unpublished) and which shows increased secretory activity during periods of hypoglycemia is probably concerned with fat and carbohydrate metabolism. That the alpha cell in the mammal is concerned with fat metabolism has been the contention of Dragstedt (1936) and Bensley and Woerner (1938).

SUMMARY

Variations in the blood glucose levels and the cells of the pancreatic islets under conditions of feeding, fasting, and the administration of insulin and alloxan were studied in several species of lizards.

The average fasting (7 to 28 days of fast) true blood glucose was 94.5 mg.%. The range was 74.0 to 113.9 mg.%. Lizards in the absorptive state had blood glucose values ranging from 140.5 to 224.1 mg.%.

A minimal shocking dose of insulin is approximately 1000 units/kg. Larger dosages of insulin (2000 to 10,500 units/kg.) did not increase the degree of shock but prolonged its duration. Hypoglycemia was observed only during the first 20 minutes after insulin injection. After 30 minutes, fasting blood sugar levels had been reattained, and after 90 minutes a compensatory hyperglycemia was invoked.

Two weeks after alloxan administration (1.5 gm./kg. given in 5 divided doses) a hyperglycemia ranging from 145.5 to 209.2 mg.% was observed coincident with destruction of beta cells.

The pancreatic islet cells of lizards stain very similarly to those of mammals and the alpha and beta cells in these two groups are probably homologous.

That the beta cells are concerned with the production of a hypoglycemic factor (insulin) is indicated by degranulation of these cells after glucose administration, and hyperglycemia subsequent to beta cell destruction by alloxan. That the alpha cells are concerned with a hyperglycemic or a fat metabolizing factor is indicated by degranulation of these cells during starvation when blood glucose levels are sub-normal and fat is being mobilized, and by granular storage during the hyperglycemia of alloxanization.

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NATURE OF THE I^{131} COMPOUNDS APPEARING IN THE THYROID VEIN AFTER INJECTION OF IODIDE-I^{131} [1,2]

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THE work of many investigators has established that the major circulating thyroid hormone is thyroxine (Taurog and Chaikoff, 1948, 1950b; Laidlaw, 1949; Rosenberg, 1951; Dingledine *et al.*, 1955; and others). Evidence for this is based primarily on the chromatographic analysis of plasma obtained from animals previously injected with I^{131}. The presence of I^{131}-triiodothyronine in such plasma has also been reported (Gross and Pitt-Rivers, 1952; Roche *et al.*, 1952a, Dingledine *et al.*, 1955) but the concentration of this component is usually very small, and in our laboratory it has, until recently, escaped detection.

In the thyroid gland itself, several iodinated amino acids are known to be present. These include monoiodotyrosine, diiodotyrosine, thyroxine, triiodothyronine, and quite possibly other iodinated compounds (Roche *et al.*, 1955a; 1955b). The iodinated amino acids are bound together, with other amino acids, in peptide linkage to form the unique iodinated protein of the thyroid gland, thyroglobulin. Release of free thyroxine into the circulation is generally considered to be the function of a specific thyroid proteolytic enzyme, which liberates the free iodinated amino acids (de Robertis, 1954; McQuillan *et al.*, 1954). Only thyroxine, and possibly triiodothyronine, are assumed to pass into the circulation; the iodotyrosines are thought to be deiodinated in the thyroid gland itself by a specific deiodinase (Roche *et al.*, 1952b).

The question may be asked whether failure to detect a given thyroid component in the general circulation necessarily implies that it is not released by the gland. In this connection it is important to recall that thyroid components such as diiodotyrosine (Tong *et al.*, 1954) and triiodothyronine (Rall *et al.*, 1953; Sterling *et al.*, 1954; Van Arsdel *et al.*, 1954, Gross, 1955) disappear very rapidly from the plasma following their injection, whereas thyroxine itself disappears much more slowly. Thus, even if I^{131}-diiodotyrosine and I^{131}-triiodothyronine were released by the thyroid gland in appreciable amounts, they would be cleared from the plasma much more rapidly than is I^{131}-thyroxine. The comparative levels of these different

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components in the general circulation, therefore, do not necessarily reflect the relative rates at which they are released by the gland.

Considerations of the above type led us to a study of the nature of the I^{131}-compounds that appear in the thyroid vein after injection of iodide-I^{131}. By sampling the venous blood draining the thyroid gland before it is diluted with the entire circulatory volume, we thought that it should be possible to determine which of the iodinated compounds in the thyroid gland are actually released into the circulation. Withdrawal of blood from the thyroid vein was facilitated by the use of large animals. Most of the work here was carried out on sheep. One horse and one dairy calf were also used as experimental animals.

MATERIALS AND METHODS

Animals—The sheep were either Suffolks, bred and raised at the University of California College of Agriculture, or Western White Face, obtained from animal dealers. The horse and the calf were stock university animals. All animals were on pasture before they were injected with I^{131} except the calf, which was still nursing.

Injection of I^{131}—Large doses of I^{131} were required to permit detection of weak components on filter paper chromatograms of plasma. Oak Ridge I^{131} was used without further purification. All injections were made via the external jugular vein with the aid of a shielded syringe operated by a crank. The sheep, weighing 50 to 90 lbs., were injected with 20–30 mc. of I^{131}. The horse, weighing 1120 lbs., received 175 mc. The calf, weighing 180 lbs., received 45 mc.

Surgical Techniques—All surgical procedures were performed in the School of Veterinary Medicine of the University of California College of Agriculture at Davis, California. At the desired time after I^{131} injection, the sheep and calf were anesthetized by intravenous injection of nembutal, and the horse with chloral hydrate. The thyroid gland, thyroid veins, and carotid artery on one side were exposed. The anatomical details varied somewhat as follows: a) *Sheep*—In the sheep, one large thyroid vein emerged from the anterior pole of the gland and entered the jugular. Several smaller veins emerged from the posterior pole of the gland. A mass ligature was first placed around the posterior veins close to their origin within the gland. The large anterior vein was ligated distally from the gland, close to its junction with the jugular, and blood was drawn from this vein directly into a heparinized syringe. Five cc. or more of thyroid venous blood were readily obtained in this manner. Immediately after the venous sample had been taken, blood was removed from the carotid artery, and both lobes of the thyroid were removed. b) *Horse*—The major thyroid vein in the horse is a fairly large vessel, approximately $\frac{1}{8}$ inch in diameter, that emerges from the posterior pole of the gland. Also visible during the experiment were a smaller anterior vein and several much smaller posterior veins. All but the large posterior vein were ligated near their origin within the gland. The major thyroid vein was ligated near its junction with the jugular, and blood was drawn from it directly into a syringe. 3) *Calf*—The arrangement of the thyroid veins in the calf was similar to that in the sheep, but the vessels were considerably smaller in diameter than those of the sheep, even though the body weight of the animal was much greater. The procedure for obtaining thyroid venous blood was similar to that described for the sheep, but considerable difficulty was encountered because of the small size of the vein.

Measurement of Uptake of I^{131} by the Thyroid Gland—Because of the large amount of radioactivity contained in the thyroid glands, it was not desirable to handle the entire gland for measurement of I^{131} uptake. A weighed aliquot of the entire gland was homog-

enized with a measured volume of bicarbonate-Ringer's solution to yield an homogenate containing approximately 50 mg. of tissue per cc. Al aliquot of this homogenate was used for I^{131}-assay. Another aliquot (0.5 cc.) was digested with 10 mg. of pancreatin for 15–18 hrs. for chromatographic analysis (Taurog *et al.*, 1955). Because of their high radioactive content, the thyroid glands were not carefully trimmed of extraneous tissue before they were weighed. The uptakes calculated in the above manner, therefore, were probably too high by several per cent.

Precipitation of Protein-Bound I^{131} of Plasma with Trichloroacetic Acid—0.5 cc. of plasma was added to 2 cc. of 20% trichloroacetic acid; the mixture was stirred well with a glass rod, and 3 cc. of 10% trichloroacetic acid were added with stirring. After centrifugation, the supernantant was transferred to a volumetric flask, and the precipitate was washed once with 5 cc. of 10% trichloroacetic acid. The supernatants were combined, and an aliquot was taken for I^{131}-assay in a well-type scintillation counter. The precipitate was dissolved in 2N NaOH, with warming, the solution was made to volume, and an aliquot was taken for counting. An appreciable percentage of inorganic I^{131} (2–3%) may remain with the trichloroacetic acid precipitate in this procedure. The chromatographic procedure described below is much more satisfactory for the detection of small percentages of protein-bound I^{131} in plasma.

Chromatographic Procedures—The techniques used for filter paper chromatography of thyroid hydrolysates and plasma have been described elsewhere (Taurog *et al.*, 1950a; Taurog *et al.*, 1955). The following two additions to the general procedures were made in the present investigation: 1) The use of a solvent consisting of butanol-ethanol-2N NH_4OH, 5:1:2. This solvent gives results similar to the butanol-dioxane-NH_4OH described by Gross and Leblond (1951). It is useful for detecting triiodothyronine (R_f 0.54–0.56), which is separates very well from thydroxine (R_f 0.42). However, it does not separate mono- and diiodotyrosine (R_f 0.11–0.13), and gives only a poor separation of thyroxine from inorganic iodide (R_f 0.36–0.38). 2) The use of concentrated butanol extracts of plasma for filter paper chromatography. In previous work we generally employed whole plasma for chromatographic analysis, rather than concentrated butanol extracts of plasma, to avoid as much as possible introducing I^{131}-artifacts into the results. We have found that much of the difficulty preveiously encountered in this regard can be eliminated by the addition of a small amount of thiouracil to butanol extracts of plasma just before they are concentrated. The details of the procedure are as follows: 1.5 cc. of plasma were extracted in a 15-cc., conical centrifuge tube, first with 3 cc., then three more times with 2 cc. of distilled butyl alcohol. The butanol extracts were separated by centrifugation and drawn off into a second tube with the aid of a capillary and suction. The combined butanol extracts were transferred to a 10-cc. beaker, and a small amount of thiouracil (0.1–0.3 mg.) was mixed in with a stirring rod. The solvent was removed at 40–50°, in a stream if nitrogen, under reduced pressure. As soon as the residue was dry, it was rubbed thoroughly with 200 or 300 μl. of 1:1 ethanol-2N NH_4OH. The resulting suspension was used directly for filter paper chromatography. Forty or 60 μl. were applied to the paper for each analysis, in 2 or 3 20-μl. portions.

Extraction of plasma-I^{131} with butanol was usually 80–85 per cent complete (except in certain samples, described below, which contained an appreciable part of their radioactivity in the form of a protein-like I^{131}-component which remained at the origin of the chromatogram). This figure agrees fairly well with the butanol extractability of synthetic preparations of I^{131}-thyroxine, I^{131}-triiodothyronine, and I^{131}-monoiodotyrosine added to plasma (Benua and Dobyns, 1955). Synthetic I^{131}-dioidotyrosine, however, was extracted from plasma only to the extent of about 55 per cent by the butanol extraction procedure used here (see also Benua and Dobyns, 1955). The relative amount of I^{131}-diiodotyrosine in a concentrated butanol extract of plasma, therefore, is somewhat less than it is in the original plasma.

For quantitative estimation of the I^{131} in the various compounds on the chromatograms, sections of the chromatograms corresponding to the bands observed on the radioautographs were cut out, folded, and placed in vials for counting in a well-type scintillation counter. The counter had a sensitivity of 10^4 counts per second per μc. of I^{131}, and a background of 1.5 counts per second. The chromatograms prepared with the collidine-water-ammonia solvent, as previously described, were used for obtaining most of the I^{131} values reported here. However, in the following instances the I^{131}-values were obtained from the butanol-ethanol-ammonium hydroxide chromatograms described above: 1) triiodothyronine in thyroid gland and plasma, and 2) total iodotyrosines in the concentrated butanol extract of plasma.

RESULTS

Body weights, thyroid gland weights, and thyroid uptakes of I^{131} are recorded in Table 1. Also shown in Table 1 are results of protein-bound I^{131}

TABLE 1. RELATIVE CONCENTRATION OF PROTEIN-BOUND I^{131} IN CAROTID ARTERY AND THYROID VEIN OF VARIOUS ANIMALS INJECTED WITH I^{131}

Animal	Weight of animal	Weight of thyroid gland	Interval after I^{131}	Uptake of I^{131} of thyroid	Protein-bound I^{131} / Total I^{131}	
					Carotid artery	Thyroid vein
	lbs.	gm.	hrs.	% of injected dose	%	%
1. Suffolk sheep	54	1.75	6	10	4.8	7.4
2. Suffolk sheep	47	1.4	26	14	22	37
3. Suffolk sheep	74	2.3	22	22	20	51
4. Suffolk sheep	90	8.3	25	50	78	87
5. Suffolk sheep	90	4.6	24	51	53	79
6. Western White Face Sheep	62	3.3	23	47	55	74
7. Western White Face Sheep	50	3.4	23	55	66	82
8. Horse	1120	43	24	8.4	8	43
9. Calf	180	4.8	20	5.6	6.3	73

studies on carotid arterial and thyroid venous plasma, as determined by trichloroacetic acid precipitation.

The chromatographic findings are summarized in Tables 2–4. Each section below deals with the chromatographic results obtained with a particular species.

A. Sheep

The results in Table 2 are based on chromatographic analyses of samples removed from sheep No. 2, Table 1, and are typical in most respects of the values obtained for the majority of the sheep. All the samples from this animal were obtained 26 hours after the injection of 29 mc. of I^{131}, at which time the total uptake of I^{131} by the thyroid was 14% of the injected dose.

Thyroid gland—The distribution of I^{131} in the hydrolyzed sheep thyroid gland was similar to that observed in the rat thyroid (Taurog *et al.*, 1955).

TABLE 2. SUMMARY OF CHROMATOGRAPHIC ANALYSIS OF THYROID GLAND, CAROTID ARTERIAL PLASMA AND THYROID VENOUS PLASMA OF SHEEP

Sheep No. 2

Fraction	Thyroid gland pancreatin digest	Thyroid artery		Thyroid vein	
		Whole plasma	Conc. butanol extract of plasma	Whole plasma	Conc. butanol extract of plasma
	% of total I^{131} in gland	cts/sec in 40 μl.	cts/sec in 40 μl.	cts/sec in 40 μl.	cts/sec in 40 μl.
Thyroxine	9.3	3.0	23.3	6.0	36.2
Triiodothyronine	1.3	neglig.	1.4	neglig.	3.1
Monoiodotyrosine	25.4	neglig.	0.5	neglig.	1.0
Diiodotyrosine	34.8	neglig.		neglig.	
Iodide	3.7	21.1	109	14.2	78.7
I^{131}-Protein*	15.8	1.0	neglig.	1.0	neglig.
Unidentified†	7.6				

* Refers to I^{131} remaining at the origin of the chromatogram. In the case of the thyroid gland this is mostly unhydrolyzed I^{131}-thyroglobulin. In the plasma samples its identity is unknown.

† Refers to very low R_f components in the thyroid digest which are probably incompletely hydrolyzed peptides of the iodinated amino acids.

Most of the I^{131} was present as mono- and diiodotyrosine (60%). Thyroxine-I^{131} accounted for approximately 10% of the total I^{131}, and the ratio $\frac{\text{triiodothyronine } I^{131}}{\text{thyroxine-}I^{131}}$ was 0.14. The presence of almost 16% of the total I^{131} at the origin indicates that an appreciable part of the original I^{131}-thyroglobulin remained unhydrolyzed after digestion with pancreatin.

Plasma from carotid artery and thyroid vein—When plasma from the carotid artery or thyroid vein of this sheep was chromatographed without preliminary treatment, only three components appeared on the radioautographs (Table 2). These were the following: 1) *Iodide-I^{131}*—This comprised the largest portion of the I^{131} in this particular animal. Iodide-I^{131} was significantly more concentrated in the artery than in the vein, indi-

TABLE 3. SUMMARY OF CHROMATOGRAPHIC ANALYSIS OF THYROID GLAND, CAROTID ARTERIAL PLASMA, AND THYROID VENOUS PLASMA OF HORSE

Fraction	Thyroid gland pancreatin digest	Thyroid artery Conc. butanol extract of plasma	Thyroid vein Conc. butanol extract of plasma
	% of total I^{131} in gland	cts/sec in 40 μl.	cts/sec in 40 μl.
Thyroxine	3.5	0.6	7.8
Triiodothyronine	0.9	neglig.	1.6
Monoiodotyrosine	35.3	neglig.	neglig.
Diiodotyrosine	35.3		
Iodide	3.3	17.1	0.4
I^{131}-Protein	14.5	neglig.	neglig.
Unidentified	6.1		

cating active uptake of iodide by the gland. 2) *Thyroxine I^{131}*—This was the major organic-I^{131} component in both artery and vein. It was twice as concentrated in the thyroid vein as in the carotid artery, indicating release of I^{131}-thyroxine from the gland into the circulation. 3) *I^{131} at the origin of the chromatogram*—This component was relatively much weaker than the others, and its identity is not known. On first thought, one might expect it to be I^{131}-thyroglobulin, released in small amounts from a thyroid gland injured by I^{131} radiation. However, the fact that it was usually present at the same concentration in both artery and vein raises the suspicion

TABLE 4. SUMMARY OF CHROMATOGRAPHIC ANALYSIS OF THYROID GLAND, CAROTID ARTERIAL PLASMA, AND THYROID VENOUS PLASMA OF CALF

Fraction	Thyroid gland pancreatin digest	Thyroid arterial plasma	Thyroid venous plasma
	% of total I^{131} in gland	cts/sec in 40 μl.	cts/sec in 40 μl.
Thyroxine	1.4	neglig.	neglig.
Triiodothyronine	0.5	neglig.	neglig.
Monoiodotyrosine	40.1	neglig.	neglig.
Diiodotyrosine	33.8	neglig.	neglig.
Iodide	3.6	48.1	43.8
I^{131}-Protein	16.5	2.7	113
Unidentified	4.0		

that it may be largely non-specific I^{131}-protein formed in extrathyroidal tissues. Another possibility is that it represents primarily an artifact of the chromatographic procedure, although we consider this unlikely.

No I^{131}-iodotyrosines and no I^{131}-triiodothyronine were detected on the chromatograms of whole plasma from the thyroid vein or the carotid artery. To increase the sensitivity of the procedure, concentrated butanol extracts of both arterial and venous plasma were prepared as described above. Portions of the concentrated extracts were chromatographed both in collidine-water ammonia and in butanol-ethanol-ammonium hydroxide. With the latter solvent and the extract of *thyroid venous plasma*, a definite band for triiodothyronine was observed on the radioautograph. It contained 8.5% of the I^{131} present in the thyroxine fraction. In the case of the extract of arterial plasma, a band for triiodothyronine was not perceptible on the radioautograph; I^{131}-assay of the triiodothyronine section of the chromatogram, however, did show a slightly higher count than background (Table 2).

The presence of very small amounts of I^{131}-iodotyrosines in the concentrated extracts of plasma was indicated by faint bands on the radioautographs. Because of the very small quantities of I^{131} involved, it is difficult to be certain that I^{131}-iodotyrosines were not formed at some stage of the chromatographic procedure. Even if the quantities found here are physiologically significant, it is possible that most, if not all of the

I^{131}-iodotyrosines present in plasma were formed in non-thyroid tissues, for in some of the sheep the concentration of I^{131}-iodotyroxines in the thyroid venous plasma did not exceed that in the carotid plasma. In any case, the difference between the concentration of I^{131}-iodotyrosines in the thyroid gland and in the thyroid vein is very striking. Certainly only a minute fraction (if any) of the iodotyrosines in the thyroid gland is released into the circulation.

B. Horse

Table 3 shows the results of chromatographic analyses on the thyroid gland, carotid arterial plasma, and thyroid venous plasma of a horse 24 hours after injection of 175 mc. of I^{131}. The total uptake by the gland was only 8% of the injected dose.

The results obtained with the horse are qualitatively similar to those described above for the sheep. The following quantitative differences may be noted: 1) The percentage of the total I^{131} in the thyroid gland present as thyroxine was much lower in the horse than in the sheep. 2) There was more triiodothyronine-I^{131} relative to thyroxine-I^{131} in the horse thyroid gland than in the sheep thyroid. 3) The concentration of thyroxine-I^{131} in the thyroid vein of the horse was at least 10 times that in the carotid arterial plasma. The A-V difference in this case, therefore, was much greater than in the sheep. 4) Triiodothyronine-I^{131} was undetectable in carotid arterial plasma of the horse even when concentrated butanol extracts were used. It was definitely detectable in the thyroid vein, however, with the same technique.

C. Calf

The chromatographic results obtained with a young dairy calf 20 hours after the injection of 45 mc. of I^{131} are summarized in Table 4. The thyroidal uptake at that time was only 5.6% of the injected dose. The results differed in certain essential respects from those described above for the sheep or the horse: 1) Thyroxine-I^{131} was present to the extent of only 1.4% of the total I^{131} in the thyroid gland, a much lower figure than that obtained for the horse or sheep. 2) No detectable thyroxine-I^{131} was present in either the carotid artery or the thyroid vein when whole plasma was chromatographed (although a faint thyroxine-I^{131} band was present on the chromatogram of the concentrated butanol extract of the thyroid venous plasma). 3) The thyroid venous plasma of the calf contained as its major I^{131}-component a thyroglobulin-like band which remained at the origin of the chromatogram and which was not extractable with butanol. A similar, but relatively much weaker, band was observed in the arterial plasma.

The presence of a thyroglobulin-like I^{131}-component in the plasma of rats treated with destructive doses of I^{131} was reported in a previous

communication (Tong *et al.*, 1952). This component was first noted 48 hrs. after the injection and was not observed in the circulation at an interval as early as 24 hours after the injection of I^{131}. Similarly, Robbins (1954) examined the serum of I^{131}-treated hyperthyroid patients four or more *days* after administration of I^{131} in order to identify thyroglobulin-I^{131} in the circulation. It is questionable, therefore, whether sufficient tissue damage had occurred in the thyroid of the calf after only 20 hours (uptake only 5.6%) to release relatively large amounts of thyroglobulin into the circulation. Unfortunately, the small amount of blood obtained from the thyroid vein of the calf precluded further study of the nature of the protein-like I^{131}-component at the origin of the chromatogram. In connection with these results, it is of interest that Larson *et al.* (1954) described, in the serum of hyperthyroid patients who received therapeutic doses of I^{131}, a non-butanol-extractable I^{131}-component which migrated with albumin in electrophoresis studies. A similar I^{131}-component in the plasma of I^{131}-treated patients has also been observed by Robbins (1955) and by Dingledine *et al.* (1955).

The formation of an I^{131}-containing component which remained at the origin of the chromatogram was also encountered in one of the sheep (No. 3, Table 1). In the thyroid vein of this animal, over 30% of the total I^{131} behaved like I^{131}-thyroglobulin. The corresponding figure for the peripheral circulation was 12%. In contrast to the calf, however, thyroxine-I^{131} was definitely detectable in both the thyroid vein and the thyroid artery of this animal.

DISCUSSION

In a previous study dealing with the nature of the I^{131}-compounds in plasma of rats injected with large doses of iodide-I^{131} (Tong *et al.*, 1952) we did not encounter (on our filter paper chromatograms) significant amounts of I^{131}-compounds other than thyroxine and inorganic iodide. Subsequent reports by Gross and Pitt-Rivers and by Roche and his co-workers, indicating that appreciable amounts of I^{131}-triiodothyronine may also be present in plasma, induced us to reinvestigate our earlier findings and to carry out further experiments on the nature of the I^{131}-compounds released into the circulation by the thyroid gland. In the present investigation we have made use of the following additions to our previously described filter paper chromatographic technique for separating the I^{131}-components of plasma: 1) A chromatographic solvent consisting of butanol-ethanol-2N NH_4OH 5:1:2, patterned after the butanol-dioxane-2N NH_4OH solvent of Gross and Leblond (1951). This solvent is especially valuable for separating triiodothyronine from thyroxine. 2) A procedure for concentrating the I^{131} in plasma involving extraction of the plasma with butanol, concentration of the extract to dryness in the presence of a small concentration of thiouracil, and solution of the residue in ammoniacal ethanol.

When the above procedures were applied to rats injected with large doses of I^{131}, we were still unable to detect triiodothyronine in plasma with certainty, although faint bands suggestive of this component were occasionally noted. The use of the butanol-ethanol-NH_4OH solvent, however, did reveal the presence of an appreciable amount of I^{131}-triiodothyronine relative to I^{131}-thyroxine in the thyroid glands of rats. This raised the question, to what extent is the I^{131}-triiodothyronine in the thyroid gland released into the circulation, and it seemed of interest, in this connection, to investigate the thyroid A-V difference in the level of I^{131}-triiodothyronine.

In all sheep and in the one horse studied here, the concentrations of I^{131}-triiodothyronine and of I^{131}-thyroxine in the thyroid vein were appreciably higher than in the carotid (and therefore presumably in the thyroid) artery. This may be taken as evidence that triiodothyronine, as well as thyroxine, is secreted by the thyroid gland into the circulation. This is in accord with the previous findings of Roche and Michel (1954), who suggest that the major source of triiodothyronine in plasma is the thyroid gland. Our results, however, do not necessarily conflict with the views of Gross and Pitt-Rivers (1953), who postulate that triiodothyronine is formed from thyroxine in the periphery.

The triiodothyronine-I^{131} in the thyroids of the animals examined here amounted to 10–20% of the thyroxine-I^{131}. These values do not necessarily indicate the ratio of stable triiodothyronine to thyroxine. Only chemical analysis can yield this information, since it is impossible in the experiments described here to be certain that the I^{131} in both of these compounds had the same specific activity. Appreciable differences in the specific activities of these compounds in human thyroids have been reported by (Braasch *et al.* (1955).

The almost complete absence of I^{131}-iodotyrosines from the thyroid veins of the animals under investigation here confirms the prevailing view that iodotyrosines are not normally released by the thyroid gland into the circulation. Even at a time when the thyroid gland contained 70% or more of its I^{131} in the form of iodotyrosines, no more than a trace of these compounds appeared in the thyroid vein. Previous failure to detect diiodotyrosine in the peripheral circulation was thought to be due to 1) lack of secretion of I^{131}-diiodotyrosine as such into the circulation, or 2) the very rapid disappearance rate of I^{131}-diiodotyrosine from plasma, known to occur in animals (Tong *et al.*, 1954). The present results indicate that the former factor is probably the more important. I^{131}-triiodothyronine likewise disappears from the periperal circulation very rapidly, but, unlike the I^{131}-iodotyrosines, it is readily detectable in the thyroid vein.

Roche and Michel and coworkers (1952b) have presented evidence that iodotyrosines are rapidly deiodinated by thyroid tissue, a process which they believe explains the failure of most investigators to detect I^{131}-iodotyrosines in the peripheral circulation. Rapid deiodination in the thy-

roid may well explain the extremely low levels of I^{131}-iodotyrosines which are present in the thyroid vein.

Benua and Dobyns (1955) reported that both I^{131}-mono- and diiodotyrosine may be present in the serum of patients treated with therapeutic doses of I^{131}. They attributed the presence of I^{131}-diiodotyrosine in serum to the damaging effects of radiation on the thyroid gland, but they concluded that the presence of I^{131}-monoiodotyrosine in serum could not be explained on this basis. Their findings differ from those of Robbins *et al.* (1952), and of Rosenberg (1951), who found no evidence of I^{131}-iodotyrosines in the serum of patients treated with large doses of I^{131}.

We recognize that large doses of I^{131} were employed in the present investigation and that some radiation damage to the thyroid may have occurred. Since the experiments were of relatively short duration, however, we do not believe that radiation damage played an essential role in our findings. Further studies will be required to determine the validity of this assumption.

SUMMARY

A study was made of the nature of the I^{131}-compounds appearing in the thyroid vein of 7 sheep, one horse, and one dairy calf, all of which had been injected with large doses of I^{131}. Thyroid venous plasma, carotid arterial plasma, and thyroid tissue, obtained simultaneously, were examined by filter paper chromatography. To detect weak I^{131}-containing components in plasma, such as triiodothyronine and iodotyrosines, concentrated butanol extracts of plasma were first prepared before chromatography. Whole plasma was always chromatographed for comparison.

I^{131}-Triiodothyronine was more readily detectable in the thyroid vein than in the peripheral circulation. In the thyroid vein, I^{131}-triiodothyronine was present to the extent of 10 to 20% of the I^{131}-thyroxine. Similar values were obtained for the ratio of the two compounds in the thyroid gland.

Both I^{131}-thyroxine and I^{131}-triiodothyronine were generally present in higher concentration in the thyroid vein than in the carotid artery, indicating that the latter compound as well as the former, is secreted by the thyroid gland into the circulation. Iodide-I^{131}, on the other hand, was always more concentrated in the artery than in the vein, indicating active uptake of iodide by the thyroid.

No I^{131}-thyroxine was detected in the thyroid vein of the calf 20 hours after I^{131} injection. Over 70% of the I^{131} in this case was in the form of a component which remained at the origin of the chromatogram, a characteristic of I^{131}-thyroglobulin or other I^{131}-proteins. A component with similar mobility was found in appreciable amounts in the thyroid vein of one sheep.

A consistent finding in all animals was the almost complete absence of I^{131}-iodotyrosines from the thyroid vein, even at times when the thyroid gland itself contained 70% or more of its I^{131} in this form. Mono- and diiodotyrosine do not leave the thyroid gland as such.

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NOTES AND COMMENTS

THE FORMATION OF I^{131}-MONOIODOTYROSINE FROM I^{131}-IODIDE BY ISOLATED PARTICULATE FRACTIONS OF NON-THYROID TISSUES[1]

It was reported recently (Taurog, *et al.*, 1955) that particulate fractions of thyroid tissue, consisting of mitochondria and microsomes, were able to form appreciable quantities of I^{131}-monoiodotyrosine when incubated with iodide-I^{131} in the absence of any added substrate. The I^{131}-monoiodotyrosine formed under these conditions was firmly bound to thyroid protein and was released as the free amino acid only after hydrolysis. A similar particulate fraction obtained from liver or kidney tissue was completely inactive in forming organic I^{131}.

Recently, we extended these studies to include particulate fractions obtained from other tissues, and we discovered that the mitochondrial-microsomal fractions derived from several *non-thyroid* tissues also possess considerable activity in converting added inorganic I^{131} to I^{131}-monoiodotyrosine. The present report deals with our findings on mammary, salivary, and spleen tissue obtained from rats.

METHODS

The chilled tissues were minced with scissors and homogenized with Krebs-Ringer bicarbonate buffer in a special tissue press recently developed in this laboratory (Emmanuel and Chaikoff, to be published). Disintegration of tissue by this procedure has been shown to result in practically complete disruption of cells with minimum damage to cell nuclei (and presumably, therefore, to other cell particulate fractions). The tissue homogenates were centrifuged at 500 $\times$g. for 10 minutes to remove nuclei, connective tissue, and tissue debris, and the supernatant extracts were then subjected to a centrifugal force of 75,000–100,000 $\times$g. for 30 minutes. The sediment, containing the mitochondrial-microsomal fraction, was suspended in Krebs-Ringer bicarbonate buffer, 1–1.5 ml. for each gm. of starting tissue. Two-ml. portions of the cell-free suspensions were incubated with carrier-free I^{131}-iodide for one or two hours at 37° with shaking. The reaction was terminated by the addition of thiouracil. Twenty-μl. portions of each sample were chromatographed before and after hydrolysis, as described previously (Taurog, *et al.*, 1955). In experiments with tissue slices, 200 mg. of tissue were incubated for one or two hours in 2.0 ml. of Krebs-Ringer bicarbonate buffer containing I^{131} and 10 γ% iodide.

RESULTS AND DISCUSSION

The relative activities of the mitochondrial-microsomal fractions derived from different tissues are shown in Table 1. It is evident that this fraction of lactating mammary, salivary, and spleen is quite active in forming I^{131}-monoiodotyrosine. As in the case of the thyroid, the I^{131}-monoiodotyrosine is formed as part of a protein and is released by enzymatic digestion. No free I^{131}-monoiodotyrosine was present in the unhydrolyzed material. In addition to I^{131}-monoiodotyrosine and I^{131}-iodide, other I^{131} components, not yet identified, were also present on the chromatograms. These will be discussed in subsequent communications. I^{131}-diiodotyrosine was present only in negligible amounts.

The formation of I^{131}-monoiodotyrosine by the mitochondrial-microsomal fractions of non-thyroid tissues is sensitive to boiling, suggesting that tissue enzymes are involved in the iodination reaction. The reaction is also sensitive to thiouracil, cyanide, and anaerobic conditions. It is not sensitive to low concentrations of catalase. In all these re-

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TABLE 1. FORMATION OF I^{131}-MONOIODOTYROSINE BY MITOCHONDRIAL-MICROSOMAL FRACTIONS FROM VARIOUS TISSUES

Tissue	Nitrogen content of mitochondrial-microsomal fraction	Per cent of added I^{131} organically bound as:	
		I^{131}-protein (before hydrolysis)	I^{131}-monoiodotyrosine (after hydrolysis)
	Mg. N per ml.		
Sheep thyroid	2.18	23.2	19.1
Rat lactating mammary	5.48	19.1	15.9
	—	29.9	23.0
Rat submaxillary	4.08	18.3	—
	—	11.2	8.2
Rat spleen	—	9.8	4.7
Rat liver	—	0.8	—

spects the behavior of the mitochondrial-microsomal fraction of the non-thyroid tissues resembles that of the thyroid. The latter, however, was readily inhibited by low concentrations of iodide, a characteristic not observed in the particulate fractions of the non-thyroid tissues. The mechanism of this inhibition requires further investigation.

When slices of the various non-thyroid tissues described here were incubated with I^{131}-iodide, none showed any ability to concentrate iodide (Table 2). Mammary slices, however, in spite of their inability to concentrate iodide, displayed definite activity in forming I^{131}-monoiodotyrosine. An iodide concentrating mechanism is not, therefore, a necessary requirement for organic iodine formation by tissue.

Previous studies indicate that I^{131}-monoiodotyrosine is the principal iodinated amino acid formed by seaweed homogenates (Tong and Chaikoff, 1955), and by xanthine-xanthine oxidase-protein systems (Roche, *et al.*, 1954; McQuillan, *et al.*, 1954). I^{131}-monoiodotyrosine (as well as diiodotyrosine) is formed by two invertebrates (a mollusk and an annelid) exposed to media containing iodide-I^{131} (Gorbman, *et al.*, 1954), and both mono- and diiodotyrosine are reported to be present in many corals (Roche, 1952), and in sponges (Roche and Yagi, 1952). Monoiodotyrosine forming activity, therefore, appears to be widespread. Apparently many tissues and tissue fractions possess enzyme systems capable of oxidizing iodide, and the resulting iodine (I_2, or possibly some higher oxidation state of iodine) is capable of iodinating tyrosine residues of proteins. It remains to be discovered whether or not the possession of a powerful iodide concentrating mechanism by intact thyroid tissue is the chief biochemical feature which differentiates it

TABLE 2. I^{131} UPTAKE AND CONVERSION TO I^{131}-PROTEIN BY TISSUE SLICES FROM VARIOUS TISSUES

Tissue	$\frac{I^{131}\text{-iodide concentration in tissue*}}{I^{131}\text{-iodide concentration in medium}}$	Per cent of added I^{131} converted to I^{131}-protein
	Avg.†	Avg.†
Sheep thyroid	55	68
Rat lactating mammary	0.9	8.0
Rat submaxillary	0.5	2.8
Rat spleen	0.2	0.6
Rat liver	0.3	0.5

* Determined in the presence of 0.001M thiouracil.
† Two or more experiments.

from many other tissues, and which explains the unique ability of the thyroid gland to form significant amounts of thyroxine under physiological conditions.

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THE ENDOCRINE SOCIETY

The 1956 Annual Meeting

The Thirty-eighth Annual Meeting of The Endocrine Society will be held in the Palmer House, Chicago, Illinois, Thursday, Friday, and Saturday, June 7–8–9, 1956.

The Committee on Local Arrangements is Dr. Norris J. Heckel as Chairman with Drs. M. Edward Davis, Sam Taylor, J. P. Greenhill, and Harley E. Cluxton as members of the Committee.

All Scientific Sessions will be held in the Palmer House. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, June 8, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Make reservations directly with the Palmer House, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

The Council requests that members send suggestions for nominations for the elective offices of the Society to the Secretary. Those desiring to do so should send this information to the Secretary immediately for transmission to the Nominating Committee.

Those wishing to present papers, which will be limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Dwight J. Ingle, 950 East 59th Street, Chicago 37, Illinois, not later than February 1, 1956. *It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that these will be presented at the meeting*—in order that they may be of reference value and suitable for printing in the program and Journals of the Society. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgements to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-

authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.

Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter perfect, since there will be no opportunity for proof reading by authors.

THE AMERICAN GOITER ASSOCIATION

The 1956 Annual Meeting

The next Annual Meeting of The American Goiter Association will be held in the Drake Hotel, Chicago, Illinois, May 3, 4 and 5, 1956.

INTERNATIONAL FERTILITY ASSOCIATION

The Second World Congress on Sterility and Fertility of the International Fertility Association will be held in Naples, Italy from May 18th to 25th, 1956. About 180 scientific papers covering all phases of infertility are scheduled to be presented. All interested physicians are invited to attend.

For all information regarding travel, registration, and hotel accommodations, write to Maxwell Roland, M.D., Chairman, Liaison Committee, 1141 Eastern Parkway, Brooklyn 13, N. Y.